Remarks

Claims 1, 3-5 and 8-12 are pending in this application. Claim 12 has been amended to change the beginning article from 'A' to 'The'. Applicants gratefully acknowledge the withdrawal of the objections to claims 4-5 and claim 10. Applicants also gratefully acknowledge the withdrawal of the 35 USC 112, second paragraph rejection of claims 1, 3-5 and 8-10. Claims 1, 3-5 and 8-12 are before the Examiner for consideration.

Amendments to the Specification and New Matter Objection

The Patent Office objects to the amendments the Applicants made in their response dated June 1, 2005 under 35 USC 132(a). Applicants amended sections of the specification by moving the sequences of paragraphs and by adding verbatim text from articles cited in the specification and incorporated by reference. Applicant submits that the verbatim addition of such text do not constitute new matter. *See* MPEP § 2163.07(b). The latest office action takes the position that the material added to the noted paragraphs constitutes new matter. Applicants respectfully traverse. Applicants provide below remarks regarding the source of amendments to the specification and submit herewith references from which information was included.

Specifically, Applicant has inserted the text of previously paragraph 0010.4 to paragraph 0010.2. Additional text from Urry et al., "Protein-Based Polymeric Materials (Synthesis and Properties), Polymeric Materials Encycolpedia, vol. 9 (1996) was included to clarify the construction of these synthetic sequences. Text from Zhang, Xiaorong, Urry, Dan; Daniell, H. "Expression of an environmentally friendly synthetic protein-base polymer gene in transgenic tobacco plants" Plant Cell Reports (1996) 16: 174 – 179 has been added to paragraph 0010.4 to clarify the construction of a $(gly\text{-}val\text{-}gly\text{-}val\text{-}pro)_{121}$ polymer.

Newly added paragraphs 0010.4.1 to 0010.4.3 consist of text derived verbatim from McPherson, David T., et al. "Production and Purification of a Recombinant Elastomeric Polypeptide, G-(VPGVG)19-VPGV, from *Escherichia coli*", Biotechnol. Prog. 1992, 8, 347-352. From Trolinder, Norma L. and Goodin, J.R. "Somatic embryogenesis and plant regeneration in cotton (*Gossypium hirsutum L.)*" Plant Cell Reports (1987) 6:231-234, Applicant has added the text identifying the suitable cultivars for regeneration after agrobacterium-mediated transformation to paragraph 0018.

Substantive Rejections

Claims 1, 3-5 and 8-12 are rejected under 35 USC §112, first paragraph for allegedly lacking enablement. Applicants respectfully traverse. The office action states that the specification "does not teach how to distinguish synthetic coding sequences from non-synthetic ones, and does not teach any pentapeptides to be other than GVGVP or VPGVG." Office Action, pages 3-4. The Examiner further states that "the instant specification fails to provide guidance for nucleic acids that comprise a synthetic coding sequence encoding any pentapeptide that is repeated at least once or nucleic acids encoding 20-251 repeats of GVGVP." Office Action, page 4.

Applicant respectfully disagrees. Applicant submits that those skilled in the art will understand what is meant by synthetic and non-synthetic without issue. According to the Merriam-Webster dictionary, "synthetic" means "of, relating to, or produced by chemical or biochemical synthesis; especially: produced artificially." A copy of the dictionary definition is submitted herewith. Applicant asserts that this plain meaning of the word applies to the instant specification and that no other differentiation between "synthetic" and "non-synthetic" is necessary. Moreover, using synthetic oligonucleotides to construct genes is a method well

known to one skilled in the art. *See* McPherson, D. et al. "Production and Purification of a Recombinant Elastomeric Polypeptide, G-(VPGVG)19-VPGV, from *Escherichia coli*", Biotechnol. Prog. 1992, 8, 347-352 and Zhang, Xiaorong, et al., submitted herewith. "Expression of an environmentally friendly synthetic protein-base polymer gene in transgenic tobacco plants" Plant Cell Reports (1996) 16: 174 – 179, submitted herewith. The availability of these publications show that those skilled in the art, at the time of this application's filing, were familiar with the construction and expression of synthetic polymers containing multiple pentapeptide repeats.

Applicants also respectfully disagrees with the statement that no guidance is given in the specification for nucleic acids that comprise a synthetic coding sequence encoding any pentapeptide that is repeated at least once or nucleic acids encoding 20-251 repeats of GVGVP. The specification provides guidance for a nucleic acid encoding GVGVP-GVGFP-GEGFP-GVGVP-GVGFP-GFGFP, which comprises two GVGVP pentapeptides. Moreover, the specification teaches that polypeptides which comprise 121 repeats of GVGVP are well known in the art. Specifically, paragraph 0010 of the specification references the publications that report expression of genes encoding the 121-mer in different systems, which content were incorporated into the specification by reference. By this amendment, Applicant has further clarified the making of a pentapeptide repeat through inserted text from cited references. For example, the elastomeric pentapeptide $(gly\text{-}val\text{-}gly\text{-}val\text{-}pro)_{10}$ may be constructed by using polymerase chain reaction.

Further examples are detailed in the specification. Line 2 of paragraph 0010 references "Hyperexpression of a Synthetic Protein-Based Polymer Gene," which was published in Methods in Molecular Biology in 1997 by Applicant's research group. This publication

provides step-by-step instructions and detailed discussions on methods and materials to construct a basic polymer building block of $(GVGVP)_{10}$ and thereafter to construct the $(GVGVP)_{121}$ polymer by ligase concatenation of the basic polymer building block. In another example, "Expression of an environmentally friendly synthetic protein-based polymer gene in transgenic tobacco plant" published in 1996 in Plant Cell Reports and cited on line 3 of paragraph 0010, similarly provides guidance on constructing the gene for $(GVGVP)_{121}$, the methods of transformation and analysis. All articles cited in paragraph 0010 were incorporated by reference and made a part of the specification. Applicant therefore asserts that the specification provides ample guidance to those skilled in the art how to construct and express pentapeptides, such as GVGVP.

Applicant thus respectfully submit that the specification, as amended, provides ample guidance for nucleic acids that comprise a synthetic coding sequence encoding any pentapeptide that is repeated at least once or nucleic acids encoding 20-251 repeats of GVGVP.

The Examiner also based her rejection of the claims because the specification did not teach a viable method of transformation. Specifically, the specification disclosed that the bombardment transformation method was identified is not suitable for the university research setting, and the agrobacterium-mediated transformation was not cultivar-independent. Applicant notes the cultivars of cotton that are useful for regeneration after agrobacterium-mediated transformation. This information was set forth in Trolinder and Goodin (1986), which had been incorporated by reference into the specification and is submitted herewith. No new matter was added. In view of this, the specification, as amended, teaches a viable method of cotton transformation.

The amended specification provides guidance to one skilled in the art to construct the synthetic polypeptide containing any number of repeats of the base GVGVP sequence and a method of transformation. Accordingly, Applicant submits that the specification is enabling, and respectfully requests that the rejection under 35 U.S.C.§112, first paragraph be withdrawn.

Claims 1, 3 5 and 8 are rejected under 35 USC § 112, first paragraph, for lacking written description. The Examiner has rejected the pending claims as containing subject matter that was not described in the specification in such as way as to reasonably convey to one skilled in the art that the inventor had possession of the claimed invention at the time the application was filed. Applicants respectfully traverse.

The office action states that the "specification describes no nucleic acid encompassed by the claims." Applicants disagree and assert that there are numerous examples of nucleic acids that are encompassed by claim 1. Applicants believe that upon considering the subject matter added in Applicants previous response, in light of the submitted references, that the Examiner will concur that there are many nucleic acids taught that are encompassed by claim 1, for example.

The office action raises the concern that one skilled in the art could not distinguish a synthetic sequence from a nonsynthetic sequence. As urged above, those skilled in the art knew well the differences between a synthetic sequences and non-synthetic (or those isolated from nature) sequences and how to make them. The difference between synthetic or non-synthetic sequences relates to the origin of such nucleic acid, i.e., whether they were produced artificially. With respect to claims, 4 and 8-12, Applicants further traverse on the grounds that based on the specific recitations of sequences in the respective claims, there can be no question that such claims meet the written description requirements. In light of the

amendments to the specification and the remarks above, Applicant respectfully requests that the 35 USC 112, first paragraph rejection of claims 1, 3-5, and 8-12 be withdrawn.

Next, claims 1, 3, 5 and 8 are rejected under 35 USC 112, first paragraph, based on lacking written description. In particular, the office action rejects the language of claim 8 "wherein said gene encodes between 20 and 251 repeats of the amino acid sequence Gly-Val-Gly-Val-Pro (SEQ ID NO:2)." Applicants respectfully traverse, and again points out that several examples of repeats in this range our disclosed in the subject application. Based on such disclosure, the range of 20-251 repeats is at least implicit if not explicit in the teachings of the subject application. Applicants respectfully request reconsideration and withdrawal of this 35 USC 112, first paragraph rejection.

Lastly, claim 12 is rejected under 35 USC 112, second paragraph, based on indefiniteness. Applicants clarify that claim 12 is dependent on claim 11. It is the synthetic gene that encodes the pentapeptide that is repeated at least once. Thus, in the context of a synthetic gene, claim 12 further defines that a synthetic gene comprises repeats of pentapeptide, and that these repeats may be present in multiples of 10, e.g., 10, 20, or 30 times etc. Applicants trust that the foregoing remarks address the rejection and respectfully request reconsideration and withdrawal of this 35 USC 112, second paragraph, rejection.

Applicant believes that this amendment has addressed all outstanding issues such that this patent application is in condition for allowance. As no issues remain outstanding, early allowance of the application is respectfully requested.

Respectfully submitted,

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CERTIFICATE OF MAILING

I HEREBY CERTIFY that this RESPONSE TO FINAL OFFICE ACTION is being deposited with the United States Postal Service as first class mail in an envelope addressed to: Commissioner for Patents, P. O. Box 1450, Mail Stop AF, Alexandria, Virginia 22313-1450 this 16th day of February 2006.

Alicia Hoffman

Plant Cell Reports (1996) 16:174–179

Plant Cell Reports

Expression of an environmentally friendly synthetic protein-based polymer gene in transgenic tobacco plants

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ABSTRACT

We report the expression of a protein-based polymer (Gly-Val-Gly-Val-Pro)$_{121}$, i. e., (GVGVP)$_{121}$ in transgenic tobacco (*Nicotiana tabacum* var. Kentucky 17) plants. The plant expression vector pBI121-XZ-120mer which contains the gene (GVGVP)$_{121}$ with a prokaryotic preferred codon composition driven by the CaMV 35S promoter was introduced into tobacco plants by *Agrobacterium*-mediated transformation. Stable integration of the (GVGVP)$_{121}$ polymer gene was confirmed by Southern blot analysis. Northern hybridization showed polymer transcripts in leaves of transgenic plants. The (GVGVP)$_{121}$ polymer protein was detected in leaves of transgenic plants by Western blot. The (GVGVP)$_{121}$ protein could be easily purified to a high degree of purity from leaves of transgenic plants by reversible phase transition as revealed by SDS-PAGE gels stained by $CuCl_2$. Transgenic plants grew, flowered, and produced seeds normally.

INTRODUCTION

Environmental problems require the development of biodegradable materials which can be produced from renewable resources without the use of toxic and hazardous chemicals, and which will help solve the increasing global solid waste disposal burden. Among such materials are protein-based polymers. Elastic and plastic protein-based polymers, defined as high polymers of repeating peptide sequences, offer a range of materials similar to that of petroleum-based polymers, such as hydrogels, elastomers, and plastics. Protein-based polymers have their origins in repeating sequences that occur in all sequenced mammalian elastin proteins (Yeh et al. 1987). In the most striking examples, the sequence (Val^1-Pro^2-Gly^3-Val^4-Gly^5)n occurs in bovine elastin with n=11, without a single substitution (Yeh et al. 1987).

Protein-based polymers can be prepared of varied design and composition and can be made biodegradable with chemical clocks to set their half lives (Urry 1995) so that they can be environmentally friendly. Protein-based polymers tested to date have been shown to have remarkable biocompatibility, thereby enabling a whole range of medical applications including the prevention of post-surgical adhesions, tissue reconstruction, and programmed drug delivery (Urry et al. 1993). For example, the polymer poly (GVGVP), used in this study, has been shown to prevent adhesions in the rat contaminated peritoneal model following abdominal injury (Urry et al. 1993). On the non-medical side, potential applications include transducers, molecular machines, superabsorbant agents, biodegradable plastics, and controlled release of agricultural crop enhancement agents, such as herbicides, pesticides, growth factors, and fertilizers.

Initially, protein-based polymers were prepared by chemical means using either classical solution syntheses or solid phase syntheses (Urry et al. 1985). As the longer repeats have begun to be designed with more functional moiety, however, the syntheses have become so difficult that genetic engineering and bioproduction becomes an increasingly attractive alternative even for research quantities of materials. Furthermore, the cost of chemical synthesis is too high, in addition to the use of undesirable noxious and hazardous chemicals.

Clearly, commercial viability of such protein-based polymers requires a cost of production that would begin to rival that of oil-based polymers. The potential to do so resides in low cost bioproduction. So far several protein-based polymers have been produced in *E. coli* through genetic engineering (McPherson et al. 1992; Krejchi et al. 1994; Urry et al. 1996; Guda et al. 1995; Daniell et al. 1996; McPherson et al. 1996). In our previous study, a synthetic polymer gene, coding for (GVGVP)$_{121}$, was hyper expressed in *E. coli* to the extent that polymer inclusion bodies occupied nearly 80-90% of the cell volume (Urry et al. 1996; Daniell 1996; Guda et al. 1995;

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Daniell et al. 1996). However, current production through fermentation is still an expensive process and not competitive with petroleum-based polymers. A possible strategy for reducing the production cost would be to produce polymers in plants, because plants are cheap to grow on a large scale. While several plant systems might be considered for polymer production, tobacco seems to be preferable because of its relative ease of genetic manipulation and an impending need to explore alternative uses for this agricultural commodity. Furthermore, production of protein-based polymers in tobacco plants will open the possibility of converting other surplus agricultural products into high value-added industrial products. In our previous study, we explored the possibility of producing protein-based polymers by expressing the $(GVGVP)_{121}$ polymer in cultured cells of tobacco (Zhang et al. 1995). In this study we investigate the potential for production of this protein-based polymer in transgenic tobacco plants and develop a polymer purification methodology from transgenic leaves.

MATERIALS AND METHODS

Construction of pBI121-XZ-121mer. The initial gene which encodes 10 repeating units of the elastomeric pentapeptide Gly-Val-Gly-Val-Pro, i. e., $(GVGVP)_{10}$ was constructed by using synthetic oligonucleotides. The gene was amplified by using polymerase chain reaction (McPherson et al. 1992). Higher molecular weight polymer genes were then made by concatenation/ligation reaction using suitable adaptor oligonucleotide fragments. Details of a series of these gene constructs have been published elsewhere (McPherson et al. 1996). These higher molecular weight polymer genes were subsequently cloned into pUC118 as a BamHI-BamHI fragment. The vector for tobacco transformation was constructed as follows. The *uid*A gene was removed from the plasmid pBI121 (Jefferson et al. 1987) as a XbaI-SstI fragment and replaced by the $(GVGVP)_{121}$ gene (obtained as XbaI-SstI fragment from the pUC118 plasmid) resulting in the construct pBI121-XZ-121mer (Fig. 1).

Tobacco transformation. Binary vector pBI121-XZ-121mer was transformed into *Agrobacterium tumafaciens* strain LBA4404 (kindly provided by Dr. E. W. Nester, University of Washington) by freeze-thaw method (An et al. 1988). The construct was introduced into *Nicotiana tabacum* var. Kentucky 17 using the leaf-disc transformation method (Horsh et al. 1985). Transgenic plants were selected on MS104 medium containing 100 mg/ml kanamycin and rooted on MSO medium containing 100 mg/ml kanamycin. Plants were then transferred to soil and analyzed. For details of media composition please see Daniell (1996).

Southern blot analysis. Total DNA was extracted from leaves of 2-week-old transformed and untransformed plants essentially as described by Rogers and Bendich (1988). Total DNA (10 μg) was digested with EcoRI, separated through a 0.7% agrose gel, and transferred to a MSI membrane (Micron Separation Inc. Westboro, MA). Prehybridization and hybridization were conducted according to Daniell et al. (1995). A ^{32}P-labeled 1.8 kb $(GVGVP)_{121}$ gene fragment was prepared by the random-primed labeling procedure (Promega) and used for hybridization.

Northern blot analysis. Total RNA was isolated from 3-week-old transformed and untransformed tobacco plants essentially as described by De Vries et al. (1988). RNA (20 μg) was denatured by formaldehyde treatment, separated in 1.2% agrose gel in the presence of formaldehyde, and transferred to MSI (Micron Separation Inc. Westboro, MA). The blot was prehybridized and hybridized as described above for Southern blot analysis.

Protein isolation and immunoblotting. Plant leaves were ground in liquid nitrogen and homogenized in one volume of the extraction buffer containing 50 mM Tris, pH 7.5, 1% 2-mecaptoethanol, 5 mM EDTA, and 2 mM phenylmethyl-sulfonyl fluoride (PMSF). Insoluble debris was removed by centrifugation at 10,000 g for 15 minutes (4°C). The pellet was discarded, and aliquots (100 μg) of the supernatant were loaded onto 10% SDS-PAGE gel according to Laemmli (1970). After electrophoresis, proteins were transferred to a nitrocellulose membrane electrophoretically in 25 mM Tris, 192 mM glycine, 5% methanol (pH 8.3). The filter was blocked with 2% dry milk in Tris-buffered saline for two hours at room temperature and stained with antiserum raised against the polymer AVGVP (kindly provided by the University of Alabama at Birmingham, monoclonal facility) overnight in 2% dry milk/Tris buffered saline. The protein bands reacting to the antibodies were visualized using alkaline phosphatase-linked secondary antibody and the substrates nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl-phosphate (Bio-Rad). Quantification of purified polymer proteins was carried out by densitometry using Scanning Analysis software (BioSoft, Ferguson, MO) installed on a Macintosh LC III computer (Apple Computer, Cupertino, USA) with a 160-Mb hard disk operating on a System 7.1, connected by SCSI interface to a Relisys RELI 2412 Scanner (Relisys, Milpitas, CA) (Wang et al. 1995). Total protein contents were determined by the dye-binding assay of Lowry et al. (1951) using reagents supplied in kit from Bio-Rad, with bovine serum albumin as a standard.

Polymer protein purification and quantitation. Plant leaves were ground in liquid nitrogen and homogenized in one volume of the extraction buffer containing 50 mM Tris-HCl, pH 7.5, 1% 2-mecaptoethanol, 5 mM EDTA, 2 mM PMSF, and 0.8 M NaCl. The homogenate was then centrifuged at 10,000 g for 10 minutes (4°C), and the pellet was discarded. The supernatant was incubated at 42°C for 30 minutes and then centrifuged immediately for 3 minutes at 5,000 g (room temperature). The pellet was resuspended in the extraction

buffer and incubated on ice for 10 minutes. The mixture was centrifuged at 12,000 g for 10 minutes (4°C). The supernatant was collected and stored at -20°C. The purified polymer protein was electrophoresed in a SDS-PAGE gel according to Laemmli (1970) and visualized by either staining with 0.3 M CuCl2 (Lee et al. 1987) or transferred to nitrocellulose membrane and probed with antiserum raised against the polymer protein as described above for Western blot analysis. Quantification of purified polymer proteins was carried out by densitometry as described above.

RESULTS

Vector construction and tobacco plant transformation. An expression vector, pBI121-XZ-120mer (Fig. 1), containing the $(GVGVP)_{121}$ coding sequence driven by the cauliflower mosaic virus (CaMV) 35S promoter and flanked by the nopline synthase (*nos*) gene terminator was made. This vector also contains the *npt*II gene driven by the *nos* promoter and flanked by the *nos* terminator to facilitate selection on kanamycin. This plasmid was introduced into tobacco plants by *Agrobacterium*-mediated transformation. Thirty five independent tobacco plants were regenerated. These plants were screened for the presence of the $(GVGVP)_{121}$ polymer gene by Southern blot analysis. Southern analysis confirmed that the $(GVGVP)_{121}$ gene was stably integrated into the genome of more than 50% of regenerated tobacco plants tested and the number of gene copies was estimated to be one to three (data not shown). However, majority of the transgenic plants contained only one copy of the integrated polymer gene.

Analysis of gene expression in transgenic tobacco leaves. Northern blot analysis was performed to determine if the $(GVGVP)_{121}$ polymer gene was transcribed in transgenic tobacco plants identified by Southern blot analysis. RNA was isolated from 3-week-old leaves of untransformed and transformed plants. Nine out of eighteen transgenic plants tested showed a hybridizing transcript of the expected size (1.8 kb) and the control untransformed did not show this band (Fig. 2). Four transgenic plants (# 10, #11, #14, and #15) were selected for further analysis. The $(GVGVP)_{121}$ polymer protein was detected by Western blot analysis with antibody raised against the polymer (AVGVP)n, which cross-reacts fairly strongly with (GVGVP)n. Expression levels were calculated from the Western blot by comparison to known amount of the $(GVGVP)_{121}$ polymer purified from *E. coli* bacterial cultures. The average protein levels were estimated to be 0.01% to 0.05% of total soluble leaf proteins (data not shown). However, these amounts should be regarded as an underestimation, since there are no ideal colorimetric assays for determination of the concentration of the $(GVGVP)_{121}$ polymer protein purified from *E. coli*, which was used as the standard. The polymer protein could not be assayed by the Bradford dye-binding procedure due to its lack of basic and aromatic amino acid residues (Bradford 1976). As with Lowry assay, color development is primarily due to the amino acids tyrosine and tryptophan, and to a lesser extent, cystine, cysteine, and histidine (Lowry et al. 1951; Peterson 1979), which are absent in the $(GVGVP)_{121}$ polymer protein. Therefore, accurate quantitation could not be obtained by the Bradford and Lowry methods using bovine serum albumin as a standard.

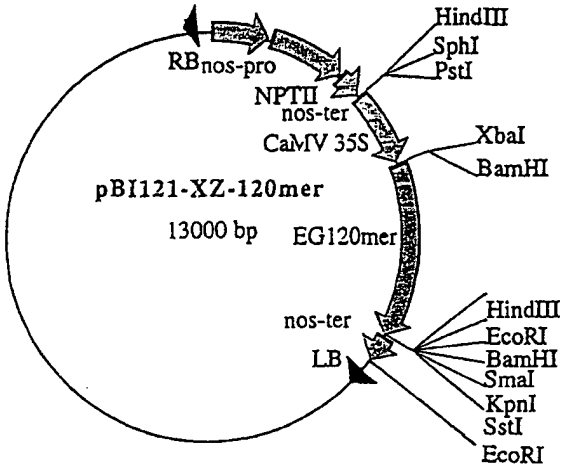

Figure 1. The plasmid pBI121-XZ-120mer. Abbreviations used in the plasmid map are: RB, T-DNA right border; nos-pro, nopline synthase gene promoter; NPTII, neomycin phosphotransferase gene that serves as a selectable marker; nos-ter; nopline synthase gene terminator; CaMV 35S, cauliflower mosaic virus 35S promoter; EG120mer, coding sequence for $(GVGVP)_{121}$; LB, T-DNA left border.

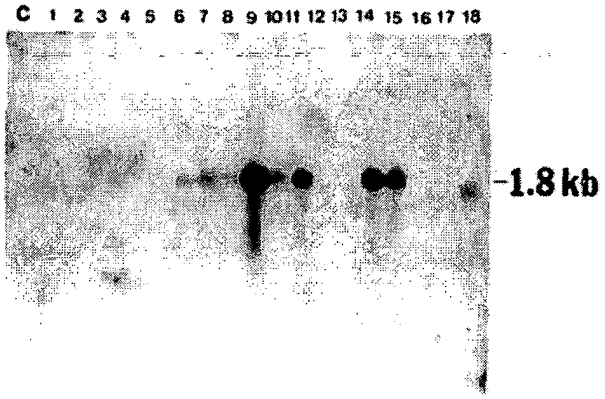

Figure 2. Northern blot analysis of tobacco leaves from control (C) and eighteen transgenic plants (1-18) using the $(GVGVP)_{121}$ gene. A transcript (1.8 kb) is evident in transgenic plants #6, #7, #8, #9, #10, #11, #14, #15, #18.

Purification of $(GVGVP)_{121}$ from transgenic tobacco leaves by temperature induced aggregation. The Polymer $(GVGVP)_{121}$ protein exhibits the property of being soluble in water at temperatures below 25°C, but aggregates into more-ordered, viscoelastic state, called a coacevate, at 37°C. This process of increasing order on increasing temperature is called an inverse temperature transition (Urry et al. 1993; McPherson et al. 1992; McPherson et al. 1996). Increasing salt concentration such as NaCl, NaBr, Na_2CO_3, and Na_3PO_4 (in order of potency) can lower the transition temperature (Urry et al. 1993). Based on this interesting and unique property, the $(GVGVP)_{121}$ and $(GVGVP)_{251}$ polymers have been purified from *E. coli* in earlier studies (Urry et al. 1994; Guda et al. 1995; Daniell et al. 1996); we report here a protocol to purify the $(GVGVP)_{121}$ polymer protein from transgenic tobacco leaves. Polymer protein purified from one gram and five grams of mature leaf is shown in Fig. 3A and Fig. 4, respectively. By introducing a single step of high temperature (42°C) treatment and including 0.8 M NaCl in the extraction buffer, high degree of purity of $(GVGVP)_{121}$ polymer protein was obtained as revealed by SDS-PAGE gels. As discussed above, polymer protein could not be visualized by Coomassie staining due to its lack of aromatic side chains and negative staining by $CuCl_2$ was used to visualize polymer protein $(GVGVP)_{121}$. The purified $(GVGVP)_{121}$ was further confirmed by Western blot analysis (Fig. 3B). The yield of the polymer protein $(GVGVP)_{121}$ was determined by comparison to a known amount of $(GVGVP)_{121}$ purified from *E. coli.* An estimated quantity of polymer protein between 0.5 μg and 5 μg could be extracted form one gram of fresh weight of leaf tissues (approximately 0.003% to 0.03% of total soluble proteins). Clearly, only a portion of polymer proteins was extractable, as compared with the quantity (0.01-0.05 % of total soluble protein) detected by the Western blot. Therefore, effort needs to be made to maximize recovery of $(GVGVP)_{121}$ from leaves by temperature-induced polymer aggregation. Demonstration of purification by inverse temperature transition attests to the successful synthesis of the $(GVGVP)_{121}$ polymer protein in tobacco plants.

Polymer $(GVGVP)_{121}$ protein was also purified from senescing leaves. The yields from old leaves were comparable to those from growing mature leaves (data not shown). This indicated that polymer protein is stable and not degraded by proteases present in plants. Apparently, there are no common protease cleavage sites in the poly GVGVP amino acid sequence.

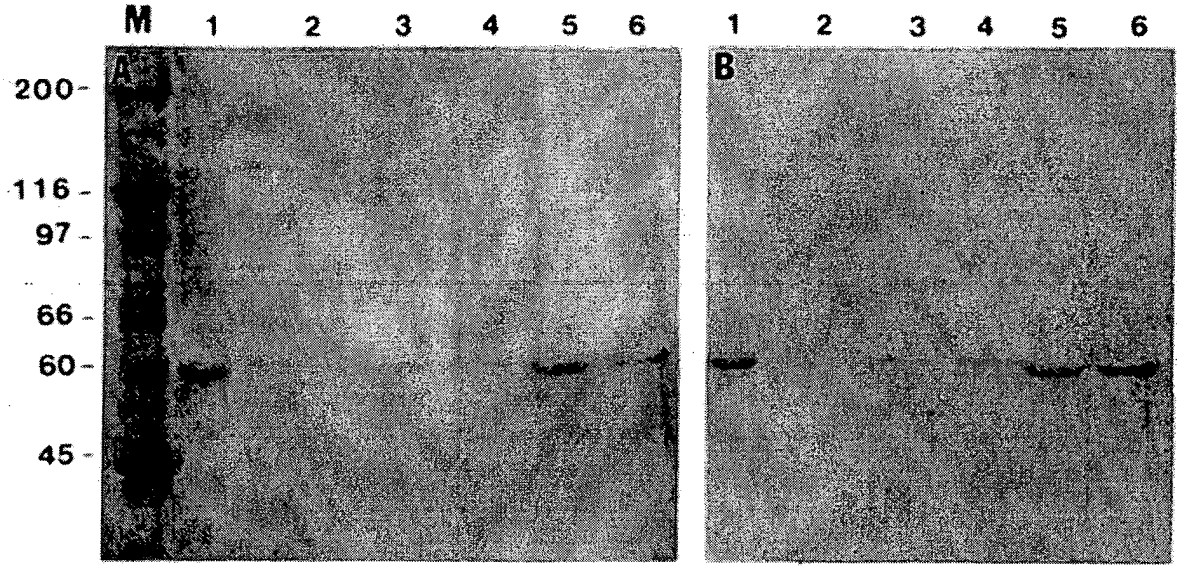

Figure 3. **A.** Copper-stained SDS-PAGE of the $(GVGVP)_{121}$ polymer protein purified from one gram (fresh weight) of leaves of plants transformed with the $(GVGVP)_{121}$ polymer gene. (M) molecular weight markers (KDa); (1) approximately 5 μg of the $(GVGVP)_{121}$ polymer protein purified from *E. coli.* (2) untransformed tobacco. (3) transgenic tobacco #10. (4) transgenic tobacco #11. (5) transgenic tobacco #14. (6) transgenic tobacco #15. **B.** Immunoblot of the SDS-PAGE identical to A. The blot was probed with an antibody raised against poly (AVGVP).

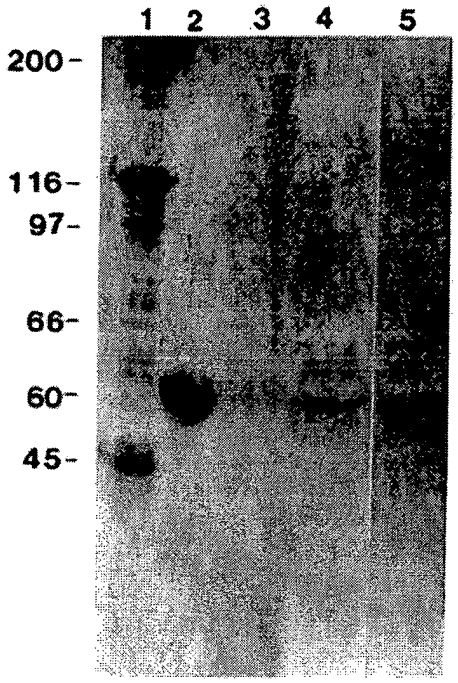

Figure 4. Copper-stained SDS-PAGE of the $(GVGVP)_{121}$ polymer protein purified from five gram (fresh weight) of leaves of plants transformed with the $(GVGVP)_{121}$ polymer gene. (1) molecular weight markers (kDa); (2) approximately 60 μg of the $(GVGVP)_{121}$ polymer protein purified from *E. coli;* (3) untransformed tobacco; (4) transgenic tobacco #11; (5) transgenic tobacco #15.

Polymer $(GVGVP)_{121}$ expressing transgenic tobacco plants did not show any phenotypic differences from untransformed plants. Transgenic plants grew, flowered and produced seeds normally. It appears, therefore, that expression of the protein-based polymer protein does not affect the growth and development of transgenic plants. Studies are in progress to further evaluate the transgenic plants and their progeny.

DISCUSSION

Plants have been used as factories for the production of biopolymers. The bacterial polyhydroxybutyrate pathway, containing three genes coding for a biodegradable thermoplastic polymer (PHB) from *Alcaligenes eutrophus*, has been successfully engineered into *Arabidopsis* plants (Nawrath et al. 1994; Poirier et al. 1992). In another study (Ebskamp et al. 1994), tobacco plants have been genetically engineered to produce fructose polymers, which have potential application in food and non-food products, by introducing a bacterial fructosyltransferase gene from *Bacillus subtilis.* However, the exact nature of the polymer produced in transgenic plants through metabolic engineering is unknown, such as the presence of monomers, molecular weight, and branching pattern of polymers, which may influence the physical properties of the end product. Unlike metabolic engineering to express other types of polymers, expression of protein-based polymer genes in this study requires the use of only a single synthetic gene and polymer proteins are direct gene products.

Another important feature of the $(GVGVP)_{121}$ polymer is its inverse temperature transition property. This makes it easier and cheaper to harvest polymers in aqueous solutions simply by raising temperature, and therefore avoid the cumbersome purification procedure and the use of enzymes and organic solvents which may alter the quality of the polymer. Extraction of $(GVGVP)_{121}$ from plants is also an important factor affecting the production cost. The ease of isolation certainly will increase the potential of $(GVGVP)_{121}$ production in plants. Yet another feature of the $(GVGVP)_{121}$ polymer is its stability, presumably because of the lack of common protease specific cleavage sites in the (GVGVP) amino acid sequence.

This study represents only the first step to produce a protein-based synthetic polymer gene product in plants. A major issue is the low level of production. High expression must be achieved in order to produce protein-based polymers on an agricultural scale. There are several reasons for the low level accumulation of the polymer protein. One possible explanation could lie in the polymer coding sequence, which has a prokaryote-preferred codon composition. Previous studies have shown that modifications of the DNA sequence of prokaryotic genes can significantly enhance expression of bacterial transgenes in higher plants (Wunn et al. 1996; Koziel et al. 1993; Perlak et al. 1991). For example, a major hurdle in engineering insect resistant plants has been the low level of expression of the *Bacillus thuringiensis* (B. t.) toxin gene; increase in B. t. toxin gene expression of up to 500-fold has been achieved through specific modification of the B. t. coding sequence to suit the eukaryotic nature of plant nuclei (Perlak et al. 1991). Therefore, a parallel expression study using the poly (GVGVP) gene with the tobacco nuclear-preferred codons is currently being conducted in our laboratory. Additionally, availability of the amino acid pool could also be a limiting factor for high level expression of this polymer gene. Earlier experiments have demonstrated that amino acid pools exist in the chloroplast (Goodwin 1971). Especially, the enzymes involved in formation of valine (Schulze-Siebert et al. 1984) and proline (Rayapati et al. 1989) are located in the chloroplast. Therefore, another possible strategy for

overcoming this problem is to change the subcellular location of $(GVGVP)_{121}$ synthesis to chloroplasts. The previous study indeed showed that the maximal level of polyhydroxybutyrate (PHB) production was increased 100-fold by changing the location of PHB production from a cellular compartment with a low flux of acetyl-CoA (cytoplasm) to a compartment with high flux of acetyl-CoA (plastid) (Nawrath et al. 1994), and the low PHB yield by cytoplasmic expression of the PHB biosynthetic enzymes could be explained by the limited supply of cytoplasmic acetyl-CoA available for PHB synthesis.

The present study demonstrated the feasibility of expressing protein-based polymers in plant systems, however, production of a large quantity of $(GVGVP)_{121}$ will require additional genetic manipulation, such as optimization of codon usage or expression of the $(GVGVP)_{121}$ polymer gene in the chloroplast compartment.

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Prostheses

See: Ligament Replacement, Artificial
Ligament Replacement Polymers (Commercial Products)
Ligament Replacement Polymers (Technological and Design Aspects)

Protective Clothing

See: Butyl Rubber (for Chemical Protective Clothing)

Protective Colloids

See: Colloids, Protective

PROTEIN-BASED POLYMERIC MATERIALS (Synthesis and Properties)

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Protein-based polymers are high polymers of repeating peptide sequences. Their potential as materials derives from the capacity to control composition and chain length, the possibility for low cost production, and an extraordinary diversity of function. They have the potential to do all that proteins do in living organisms (this is a realizable potential as the principles of folding, assembly, and function become adequately understood), and they can be designed to do more than evolution has yet called upon proteins to do.

Through evolution proteins were never called upon to prevent adhesions after surgical procedures; to prevent adhesion to catheters and drainage tubes used in medical procedures; to function in soft tissue augmentation for cosmetic, reconstructive, and other reasons; to provide functional scaffoldings that could be remodeled into natural tissues; to provide matrices for controlled drug delivery; to function as biosensors in various assays; to function as transducers capable of both sensing and actuating, that is, interconverting free energies involving the intensive variables of temperature, pressure, mechanical force, chemical potential, electro-chemical potential and electromagnetic radiation; to be biodegradable plastics with controllable half-lives; to be controllable super absorbents; to function in controlled release of agricultural enhancement factors, and so on. These examples and many more are possible uses for protein-based polymeric materials.[1,2]

This report presents specific examples of chemical and biological syntheses of protein-based polymers and the discussion of properties that demonstrate the importance of accurate control of primary structure. The capacity to produce polymers capable of performing many forms of free energy transduction indicates the state of our understanding of design principles.

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PROTEIN-BASED POLYMER SYNTHESIS

Protein-based polymers may be synthesized using classical solution and solid phase chemical methods and using gene construction and expression in the cells of animals and plants. Both approaches are discussed below with explicit characterization of products whose properties demonstrate the importance of optical purity and precise control of amino acid sequence.

Chemical Syntheses

The significant care required for correct and reproducible chemical syntheses will be briefly discussed followed by the detailed solution syntheses of composite pentamers, namely, their incorporation into 30mers of exact sequence that are then polymerized to form polytricosapeptides, or poly (30mers) of fixed sequence. The composite pentamers are also randomly mixed in the appropriate ratios and polymerized in an attempt to obtain polymers of the same or similar average composition but without fixed sequence. In this way, it becomes apparent that the properties necessary to achieve efficient function require absolute control of sequence.

Synthesis of (Gly-Val-Gly-Val-Pro) and Poly(GVGVP)

The classical solution syntheses of (GVGVP) and poly(GVGVP) illustrate the care required to achieve the property most central to the function of these protein-based polymers and possibly of proteins themselves. The functional property is the temperature, T_t, at which the hydrophobic folding and assembly transition occurs as the temperature is raised through the transition range.

The detailed synthesis of the pentamer, (GVGVP), is given below, followed by the syntheses of other pentamers, of the tricosapeptides (30mers), and of the polytricosapeptides, poly(30mers). Here the importance of purity, particularly optical purity, of the components of the pentamer is stressed. The pentamer synthesis goes forward by a dimer plus trimer strategy. The dimer, VP, is synthesized, carefully purified, and crystallized. The synthesis of the trimer, GVG, occurs by synthesizing, purifying, and crystallizing VG and then adding G to give GVG, which is purified and coupled to VP to give GVGVP. In general, on activation of the carboxyl to give the peptide bond, racemization can occur. Since Gly is not optically active, it cannot racemize; and because of the cyclic side-chain of Pro, this residue does not appreciably racemize. Thus, racemization is a problem during the coupling to form VP and VG, and the purification and crystallization of these dipeptides are critical to the optical purity of the pentamer. With activation of the Pro of GVGVP, the polymerization of GVGVP can proceed with an expectation of an optically pure, protein-based polymer.[3,4]

The sensitivity of the value of T_t to polymer purity is seen in Figure 1 The T_t values for poly(GVGVP) in Figure 1A vary from 25° to 38 °C, yet the nuclear magnetic resonance (NMR) spectra for several of the syntheses in Figure 1B provide no significant reason for the different values of T_t. There is a systematic difference, however, seen in the corresponding optical rotatory dispersion (ORD) spectra of Figure 1C. The results of Figure 1 suggest that racemization is responsible for the important differences in T_t. Comparison with microbially synthesized $(GVGVP)_n$ where racemization does not occur and for this case where n = 251 confirms the absence of racemization for the low values of T_t. The values of T_t are reasonable; the NMR spectra are as required for the microbially prepared polymers, and the ORD spectra show the correct chemical syntheses to be those with T_t values approaching 25 °C. Fortunately, chemical syntheses with the lower values of T_t had previously been deduced to be the correct syntheses. The slightly lower value of T_t of 25.8 °C for the best chemical syntheses as compared to the 26.1 °C value for the microbial product is due to the molecular weight dependence of T_t. SDS-polyacrylamide gels show the spread of molecular weight for the chemical syntheses with a T_t of 25.8 °C to occur at higher molecular weights than for the *E. coli* prepared sample which is 251 × 409 ≈ 105,660 Da. Producing such high molecular weight protein-based polymers by chemical syntheses, with average chain lengths of more than 1255 residues, represents a significant synthetic achievement.

Syntheses of the Pentamers Forming the Tricosapeptides

The syntheses of the pentamers forming the tricosapeptides, (GEGFP GVGVP GVGFP GFGFP GVGVP GVGFP), (GEGFP GVGVP GVGVP GVGVP GFGFP GFGFP), (GEGVP GFGFP GFGVP GVGVP GFGFP GVGVP), (GDGFP GVGVP GVGFP GFGFP GVGVP GVGFP), (GDGFP GVGVP GVGVP GVGVP GFGFP GFGFP), AND (GDGVP GFGFP GFGVP GVGVP GFGFP GVGVP) follow:

Boc-VP-OBzl (I): Boc-V-OH (Boc: tert-butyloxycarbonyl; V: valine; 56.18 g, 0.30 mol) was dissolved in acetonitrile (800 ml) and cooled to 0 °C and N-methylmorpholine (NMM; 33.0 ml) was added. The solution was cooled to −15° ± 1 °C and isobutylchloroformate (IBCF; 41.07 ml) was added slowly under stirring while maintaining the temperature at −15 °C.[5,6] After stirring the reaction mixture for 10 min at this temperature, 1-Hydroxybenzotriazole (HOBt; 40.63g) was added.[7] The reaction

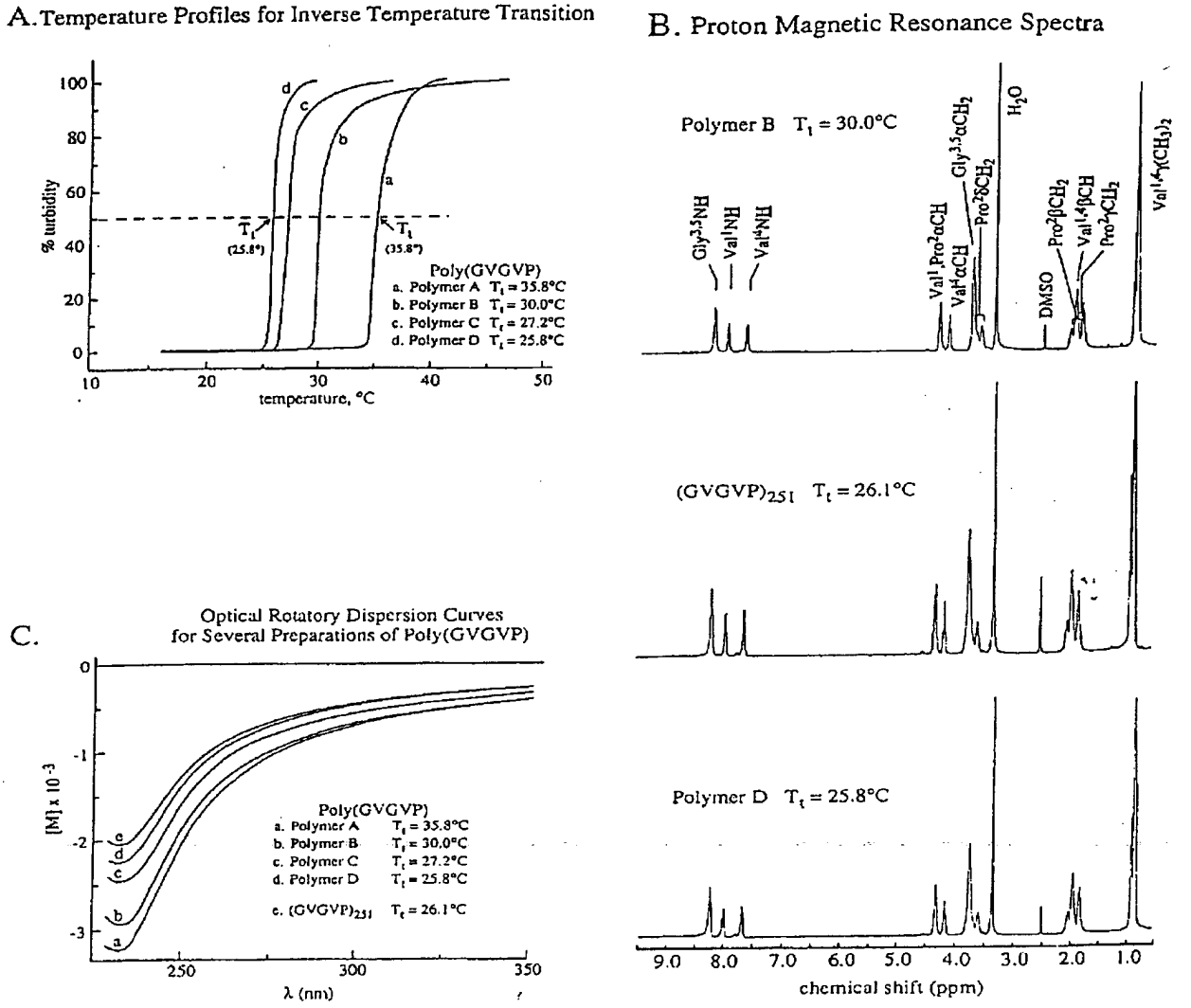


FIGURE 1. (A) Temperature profiles for the inverse temperature transition to determine T_t for the particular polymer. The polymers A, B, C, and D are different syntheses of poly(GVGVP), all having been dialyzed against 50,000 mw cut-off membranes, but each giving a different value of T_t. (B) Proton magnetic resonance spectra of polymer B, microbially synthesized $(GVGVP)_{251}$ and chemically synthesized polymer D. (C) Optical rotatory dispersion curves for the several chemical syntheses of poly(GVGVP) in Part A having different T_t values, showing essentially indistinguishable NMR curves as in Part B, but exhibiting systematic differences in molar ellipticity. This suggests the different T_t values are due to small amounts of racemization of the Val residues during chemical syntheses. This is substantiated by the microbially prepared $(GVGVP)_{251}$, which would have no racemization.

mixture was stirred for an additional 10 min and a pre-cooled solution of HCl·H—P—OBzl (P: proline; OBzl: benzylester; 72.51g, 0.30 mol) and NNM (33.0 ml) in dimethylformamide (DMF; 400 ml) was slowly added. After 20 min, the pH of the solution was adjusted to 8 by the addition of NMM and the reaction was continued overnight. The solvent was removed under reduced pressure and the residual DMF was poured into 2000 ml of ice-cold 90% saturated $KHCO_3$ solution and stirred for 30 min. The precipitant was filtered and washed with water, 10% citric acid and water. The resulting material was recrystallized from ether/petroleum ether and dried to obtain 88.5g (yield 72.92%) of I.

Boc-VG-OBzl (II): Boc-V-OH (94.80 g, .4363 mol) was dissolved in acetonitrile (1000 ml) and cooled to 0 °C. NMM (47.97 ml) was added and the solution was cooled to −15° ± 1 °C. IBCF (56.59 ml) was added slowly under stirring while maintaining the temperature at −15 °C. After stirring the reaction mixture for 20 min, a pre-cooled solution of Tos·G—OBzl (G: glycine; Tos: p-Tosylate; 147.21 g, 0.4363 mol) and NMM (47.97 ml) in DMF (500 ml) was slowly added. After 20 min, the pH of the solution was adjusted to 8 by the addition of NMM and the reaction was continued overnight. The solvent was removed under reduced pressure and the residual DMF was poured into 2000 ml of ice-cold 90% saturated $KHCO_3$ solution and stirred for 30 min. The precipitant was filtered and washed with water, 10% citric acid, and water. The resulting material was recrystallized from ethyl acetate/petroleum ether and dried to obtain 115.3 g (yield 72.51%) of II.

Boc-GVG-OBzl (III): Compound (Compd) II (112.12 g, 0.3077 mol) was deprotected by stirring for 1.5 h in 4.0 N HCl in dioxane. Excess HCl and dioxane were removed under reduced pressure, triturated with ether, washed with ether, and dried. Boc-G-OH (53.90 g, 0.3077 mol) in DMF was coupled to the above hydrochloride salt using the IBCF method described in Compd II to obtain 112.3 g (yield 86.60%) of III.

Boc-GVG-OH (IV): Compd III (112.0 g, 0.2657 mol) was dissolved in glacial acetic acid (1000 ml), and 11.2 g of 10% Palladium on Activated Charcoal (Pd/C) was added. This mixture was hydrogenated overnight at 40 psi of hydrogen (H_2) gas. The reaction mixture was filtered through celite and the solvent was removed under reduced pressure. The resulting residue was triturated with ether, filtered, washed with ether and, dried to obtain 81.2 g (yield 92.22%) of IV.

Boc-GVGVP-OBzl (V): Compd I (67.66 g, 0.1672 mol) was deprotected with HCl/dioxane and coupled to Compd IV (55.42 g, 0.1672 mol) using the IBCF method described in Compd II. Instead of recrystallization, the material was washed with ether and dried to obtain 80.0 g (yield 77.43%) of V.

Boc-FP-OBzl (VI): Boc-F-OH (F: phenylalanine; 79.30 g, 0.2989 mol) was coupled to HCl·P—OBzl (72.24 g, 0.2989 mol) using the IBCF with HOBt method described in Compd I. Since the peptide did not precipitate out, it was extracted into $CHCl_3$, which was washed with water, 10% citric acid, water, and dried over Na_2SO_4. The solvent was removed under reduced pressure. The resulting oil was recrystallized from ether/petroleum ether and dried to obtain 109.6 g (yield 81.02%) of VI.

Boc-FG-OBzl (VII): Boc-F-OH (35.56 g, 0.1340 mol) was coupled to Tos·H—G—OBzl (45.22 g, 0.1340 mol) using the IBCF method described in Compd II. Since the peptide did not precipitate out, it was taken into $CHCl_3$ and extracted. The solvent was removed under reduced pressure and the resulting oil was dried to obtain 47.8 g (yield 86.46%) of VII.

Boc-GFG-OBzl (VIII): Compd VII (45.04 g, 0.1092 mol) was deprotected with HCl/dioxane and coupled to Boc-G-OH (19.13 g, 0.1092 mol) using IBCF. Since the peptide did not precipitate out, it was taken into $CHCl_3$ and extracted. The solvent was removed under reduced pressure, and the resulting residue was triturated with ether, filtered, washed with ether, and dried to obtain 44.2 g (yield 86.21%) of VIII.

HCl·H·FP-OBzl (IX): Compd VI (100.54 g, 0.2221 mol) was deprotected by stirring for 1.5 h in 4.0 N HCl in dioxane. The excess HCl and dioxane were removed under reduced pressure, triturated with ether, washed with ether, and dried to obtain 86.4 g (yield 100%) of IX.

Boc-GFGFP-OBzl (X): Compd VIII (43.84 g, 0.0934 mol) was hydrogenated and coupled to Compd IX (36.32 g, 0.0934 mol) using IBCF. Since the peptide didn't precipitate out, it was taken into $CHCl_3$ and extracted. The solvent was removed under reduced pressure and the resulting residue was triturated with ether, washed with ether, and dried to obtain 60.7 g (yield 91.06%) of X.

Boc-GVGFP-OBzl (XI): Compd IV (22.89 g, 0.0691 mol) was coupled to Compd IX (26.86 g, 0.0691 mol) using IBCF. Instead of using recrystallization at the final step, the material was washed with ether and dried to obtain 40.3 g (yield 87.63%) of XI.

HCl·H·VP-OBzl (XII): Compd I (35.7 g, 0.0883 mol) was deprotected using HCl/dioxane as described in Compd IX to obtain 30.08 g (yield 100%) of XII.

Boc-GFGVP-OBzl (XIII): Compd VIII (7.04 g, 0.0150 mol) was hydrogenated and coupled to Compd XII (5.1 g, 0.0150 mol) using IBCF to obtain 9.4 g (yield 94.0%) of XIII.

Boc-E(OCHx)G-OBzl (XIV): Boc-E(OCHx)-OH (E: glutamic acid; OCHx: cyclohexyl ester; 16.47 g, 0.050 mol) was dissolved in 500 ml of DMF and cooled to −15 °C. HOBt (6.76 g, 0.050 mol) and 1-ethyl-3-(3-dimethylamino-propyl) carbodiimide (EDCI: 9.59 g, 0.050 mol) were

added.[8] After 20 min, a pre-cooled solution of Tos·H-G—OBzl (16.87 g, 0.050 mol) and NMM (5.5 ml, 0.05 mol) in DMF was added. The reaction mixture was stirred overnight at room temperature. The DMF was removed under reduced pressure and the residue was extracted into $CHCl_3$. The $CHCl_3$ extract was washed with water, 10% citric acid, 5% $NaHCO_3$, water, and dried over Na_2SO_4. The solvent was removed under reduced pressure and dried to obtain 22.2 g (yield 93.16%) of XIV.

Boc-GE(OCHx)G-OBzl (XV): Compd XIV (15.58 g, 0.0327 mol) was deprotected using HCl/dioxane and coupled to Boc-G-OH (5.73 g, 0.0327 mol) using the EDCI with HOBt. The reaction was worked up by extractions to obtain 16.0 g (yield 91.69%) of XV.

Boc-GE(OCHx)GFP-OBzl (XVI): Compd XV (16.00 g, 0.030 mol) was hydrogenated and coupled to Compd IX (11.76 g, 0.030 mol) using EDCI with HOBt. The reaction was worked up by extractions to obtain 20.66 g (yield 88.52%) of XVI.

Boc-GE(OCHx)GVP-OBzl (XVII): Compd XV (12.86 g, 0.0241 mol) was hydrogenated and coupled to Compd XII (8.2 g, 0.0241 mol) using EDCI with HOBt. The reaction was worked up by extractions to obtain 15.2 g (yield 86.76%) of XVII.

Boc-D(OCHx)G-OBzl (XVIII): Boc-D(OCHx)-OH (D: aspartic acid; 4.34 g, 0.0138 mol) was coupled to Tos·H-G-OBzl (4.65 g, 0.0138 mol) using EDCI with HOBt. The reaction was worked up by extractions to obtain 6.0 g (yield 94.19%) of XVIII.

Boc-GD(OCHx)G-OBzl (XIX): Compd XVIII (4.97 g, 0.0107 mol) was deprotected with HCl/dioxane and coupled to Boc-G-OH (1.88 g, 0.0107 mol) using EDCI with HOBt. The reaction was worked up by extractions to obtain 4.8 g (yield 86.00%) of XIX.

Boc-GD(OCHx)GFP-OBzl (XX): Compd XIX (4.68 g, 0.009 mol) was hydrogenated and coupled to Compd IX (3.5 g, 0.0090 mol) using EDCI with HOBt. The reaction was worked up by extractions to obtain 6.88 g (yield 100%) of XX.

Boc-GD(OCHx)GVP-OBzl (XXI): Compd XIX (4.62 g, 0.0089 mol) was hydrogenated and coupled to Compd XII (3.02 g, 0.0089 mol) using EDCI with HOBt. The reaction was worked up by extractions to obtain 6.0 g (yield 94.28%) of XXI.

Boc-GVGVP-OH (XXII): Compd V (65.45 g, 0.1060 mol) was dissolved in glacial acetic acid and hydrogenated, in the presence of 10% Pd/C overnight at 40 psi of H_2 gas. After the catalyst and acetic acid were removed, the resulting residue was taken in 5% $NaHCO_3$. The solution was washed 3X with $CHCl_3$. The pH of the solution was adjusted to pH 2 and NaCl was added until it precipitated. The solution was then extracted 3X with $CHCl_3$. The $CHCl_3$ was removed under reduced pressure. The resulting residue was triturated with ether, filtered, washed with ether, and dried to obtain 47.2 g (yield 84.43%) of XXII.

Boc-GFGFP-OH (XXIII): Compd X (46.73 g, 0.0702 mol) was hydrogenated and worked up by extractions to obtain 35.2 g (yield 82.89%) of XXIII.

Boc-GD(OCHx)GFP-OH (XXIV): Compd XX (6.88 g, 0.0090 mol) was hydrogenated and worked up by extractions to obtain 6.1 g (yield 100%) of XXIV.

Boc-GE(OCHx)GFP-OH (XXV): Compd XVI (18.86 g, 0.0242 mol) was hydrogenated and worked up by extractions to obtain 16.2 g (yield 97.15%) of XXV.

Boc-GE(OCHx)GVP-OH (XXVI): Compd XVII (14.2 g, 0.0208 mol) was hydrogenated and worked up by extractions to obtain 13.2 g (yield 99.09%) of XXVI.

Boc-GD(OCHx)GVP-OH (XXVII): Compd XXI (6.0 g, 0.0084 mol) was hydrogenated and worked up by extractions to obtain 4.2 g (yield 80.15%) of XXVII.

Boc-GVGVP-ONp (ONp: p-nitrophenyl ester) (XXVIII): Compd XXII (14.72 g, 0.0279 mol) was reacted with bis(4-nitrophenyl carbonate) (bis-PNPC; 1.5 equiv) in Pyridine (100 ml).[9] When the reaction was complete as determined by tlc, the solvent was removed under reduced pressure. The residue was worked up by extractions. The solvent was removed under reduced pressure, triturated with ether, filtered, washed with ether, and petroleum ether and dried to obtain 20.8 g (yield 93.10%) of XXVIII.

Boc-GVGFP-ONp (XXIX): Compd XI (11.39 g, 0.0171 mol) was hydrogenated and reacted with bis-PNPC and worked up by extractions to obtain 8.6 g (yield 72.03%) of XXIX.

Boc-GFGFP-ONp (XXX): Compd XXIII (14.61 g, 0.0234 mol) was reacted with bis-PNPC and worked up by extractions to obtain 12.2 g (yield 69.93%) of XXX.

Boc-GD(OCHx)GFP-ONp (XXXI): Compd XXIV (3.1 g, 0.0046 mol) was reacted with bis-PNPC and worked up by extractions to obtain 3.2 g (yield 87.43%) of XXXI.

Boc-GE(OCHx)GFP-ONp (XXXII): Compd XXV (4.13 g, 0.0060 mol) was reacted with bis-PNPC and worked up by extractions to obtain 4.2 g (yield 86.41%) of XXXII.

TFA·GVGVP-OBzl (XXXIII): Compd V (13.95 g, 0.0225 mol) was dissolved in Trifluoroacetic acid (TFA) (120 ml) and stirred for 45 min. The TFA was evaporated and the resulting residue was triturated with ether, filtered, washed with ether, and dried to obtain 15.9 g (yield 100%) of XXXIII.

TFA·GFGFP-OBzl (XXXIV): Compd X (16.75 g, 0.0235 mol) was deprotected using TFA and washed with ether to obtain 19.1 g (yield 100%) of XXXIV.

TFA·GVGFP-OBzl (XXXV): Compd XI (128.33 g, 0.0030 mol) was deprotected using TFA and washed with ether to obtain 32.3 g (yield 100%) of XXXV.

The above constitutes the detailed syntheses of the required pentamers that are placed in correct order to obtain the desired tricosamers (30mers) below.

Tricosapeptides (Hexamer of Pentamers), Alignment of Six Varied Pentamers

Boc-GVGVP GVGVP-OBzl (XXXVI): Compd XXII (8.44 g, 0.0160 mol) was coupled to Compd XXXIII (15.88 g, 0.0160 mol) using EDCI with HOBt. The reaction was worked up by extractions to obtain 15.3 g (yield 93.07%) of XXXVI.

Boc-GFGFP GVGVP-OBzl (XXXVII): Compd XXIII (6.24 g, 0.010 mol) was coupled to Compd XXXIII (6.3 g, 0.010 mol) using EDCI with HOBt. The reaction was worked up by extractions to obtain 8.6 g (yield 76.58%) of XXXVII.

Boc-GFGFP GFGVP-OBzl (XXXVIII): Compd XIII (6.7 g, 0.010 mol) was deprotected by TFA and coupled to XXIII (6.24 g, 0.010 mol) using EDCI with HOBt. The reaction was worked up by extractions to obtain 8.6 g (yield 76.58%) of XXXVIII.

Boc-GVGVP GVGFP-OBzl (XXXIX): Compd XXII (15.79 g, 0.0299 mol) was coupled to Compd XXXV (32.27 g, 0.0299 mol) using EDCI with HOBt. The reaction was worked up by extractions to obtain 30.41 g (yield 94.52%) of XXXIX.

Boc-GFGFP GFGFP-OBzl (XL): Compd XXIII (10.22 g, 0.0164 mol) was coupled to Compd XXXIV (19.07 g, 0.0164 mol) using EDCI with HOBt. The reaction was worked up by extractions to obtain 17.1 g (yield 85.61%) of XL.

Boc-GFGFP GVGVP GVGFP-OBzl (XLI: Compd XXXIX (14.06 g, 0.0131 mol) was deblocked with TFA and coupled to Compd XXIII (8.16 g, 0.0131 mol) using EDCI with HOBt. The reaction was worked up by extractions to obtain 18.6 g (yield 89.96%) of XLI.

Boc-GVGVP GFGFP GFGFP-OBzl (XLII): Compd XL (16.51 g, 0.0135 mol) was deblocked with TFA and coupled to Compd XXII (7.14 g, 0.0135 mol) using EDCI with HOBt. The reaction was worked up by extractions to obtain 18.1 g (yield 82.06%) of XLII.

Boc-GVGVP GFGFP GVGVP-OBzl (XLIII): Compd XXXVII (8.6 g, 0.0077 mol) was deblocked with TFA and coupled to Compd XXII (4.04 g, 0.0077 mol) using EDCI with HOBt. The reaction was worked up by extractions to obtain 10.1 g (yield 86.10%) of XLIII.

Boc-GE(OCHx)GFP GVGVP GVGFP-OBzl (XLIV): Compd XXXIX (9.14 g, 0.0085 mol) was deblocked with TFA and coupled to Compd XXV (5.85 g, 0.0085 mol) using EDCI with HOBt. The reaction was worked up by extractions to obtain 9.5 g (yield 67.86%) of XLIV.

Boc-GE(OCHx)GFP GVGVP GVGVP-OBzl (XLV): Compd XXXVI (8.5 g, 0.0083 mol) was deblocked with TFA and coupled to Compd XXV (5.69 g, 0.0083 mol) using EDCI with HOBt. The reaction was worked up by extractions to obtain 10.4 g (yield 78.73%) of XLV.

Boc-GE(OCHx)GVP GFGFP GFGVP-OBzl (XLVI): Compd XXXVIII (1.46 g, 0.0023 mol) was deblocked with TFA and coupled to Compd XXVI (2.69 g, 0.0023 mol) using EDCI with HOBt. The reaction was worked up by extractions to obtain 3.1 g (yield 80.31%) of XLVI.

Boc-GD(OCHx)GFP GVGVP GVGFP-OBzl (XLVII): Compd XXXIX (2.26 g, 0.0021 mol) was deblocked with TFA and coupled to Compd XXIV (1.42 g, 0.0021 mol) using EDCI with HOBt. The reaction was worked up by extractions to obtain 2.9 g (yield 84.3%) of XLVII.

Boc-GD(OCHx)GFP GVGVP GVGVP-OBzl (XLVIII): Compd XXXVI (1.85 g, 0.0018 mol) was deblocked with TFA and coupled to Compd XXIV (1.2 g, 0.0018 mol) using EDCI with HOBt. The reaction was worked up by extractions to obtain 2.2 g (yield 78.01%) of XLVIII.

Boc-GD(OCHx)GVP GFGFP GFGVP-OBzl (XLIX): Compd XXXVIII (2.69 g, 0.0023 mol) was deblocked with TFA and coupled to Compd XXVII (1.42 g, 0.0023 mol) using EDCI with HOBt. The reaction was worked up by extractions to obtain 3.1 g (yield 81.15%) of XLIX.

TFA·GFGFP GVGVP GVGFP-OBzl (L): Compd XLI (17.23 g, 0.0109 mol) was deprotected using TFA and washed with ether to obtain 17.5 g (yield 99.9%) of L.

TFA·GVGVP GFGFP GFGFP-OBzl (LI): Compd XLII (17.12 g, 0.0105 mol) was deprotected using TFA and washed with ether to obtain 17.3 g (yield 99.8%) of LI.

TFA·GVGVP GFGFP GVGVP-OBzl (LII): Compd XLIII (10.1 g, 0.0066 mol) was deprotected using TFA and washed with ether to obtain 10.2 g (yield 100%) of LII.

Boc-GE(OCHx)GFP GVGVP GVGFP GFGFP GVGVP GVGFP-OBzl (LIII): Compd XLIV (8.88 g, 0.0054 mol) was hydrogenated and coupled to Compd L (8.6 g, 0.0054 mol) using EDCI with HOBt. The reaction was worked up by extractions to obtain 15.0 g (yield 93.1%) of LIII.

Boc-GEGFP$(GVGVP)_3(GFGFP)_2$-OBzl (LIV)-Compd XLV (9.58 g, 0.006 mol) was hydrogenated and coupled to Compd LI (9.86 g, 0.006 mol) using EDCI with HOBt. The reaction was worked up by extractions to obtain 17.5 g (yield 97.11%) of LIV.

Boc-GEGVP GFGFP GFGVP GVGVP GFGFP GVGVP-OBzl (LV): Compd XLVI (2.71 g, 0.0016 mol) was hydrogenated and coupled to Compd LII (2.41 g, 0.0016 mol) using EDCI with HOBt. The reaction was worked up by extractions to obtain 4.0 g (yield 85.15%) of LV.

Boc-GD(OCHx)GFP GVGVP GVGFP GFGFP GVGVP GVGFP-OBzl (LVI) Compd XLVII (2.77 g, 0.0017 mol)

was hydrogenated and coupled to Compd L (2.7 g, 0.0017 mol) using EDCI with HOBt. The reaction was worked up by extractions to obtain 5.06 g (yield 100%) of LVI.

Boc-GD(OCHx)GFP(GVGVP)$_3$(GFGFP)$_2$-OBzl (LVII): Compd XLVIII (2.2 g, 0.0014 mol) was hydrogenated and coupled to Compd LI (2.3 g, 0.0014 mol) using EDCI with HOBt. The reaction was worked up by extractions to obtain 4.16 g (yield 100%) of LVII.

Boc-GD(OCHx)GVP GFGFP GFGVP GVGVP GFGFP GVGVP-OBzl (LVIII): Compd XLIX (2.69 g, 0.0016 mol) was hydrogenated and coupled to Compd LII (2.53 g, 0.0016 mol) using EDCI with HOBt. The reaction was worked up by extractions to obtain 4.4 g (yield 89.07%) of LVIII.

The preceding provides the detailed chemical syntheses of the desired tricosamers (30mers), which will be polymerized as described below.

Polymerization of Tricosapeptides

Poly[GDGFP GVGVP GVGFP GFGFP GVGVP GVGFP] (LIX): Compd LVI (4.21 g, 0.0014 mol) was hydrogenated and washed with ether. The material was then deprotected with TFA. A 0.1 molar solution of the TFA salt in DMSO was polymerized for 18 days using EDCI with HOBt and 1.6 equiv of NMM as base. The polymer was placed in water, dialyzed using 3500 mol. wt. cut-off tubing and lyophilized. The side chain was then deprotected using HF: p-cresol (90:10, v/v) at O °C for 1 h. [10,11] It was triturated with ether and then dissolved in water, dialyzed using 50,000 mol. wt. cut-off tubing, and lyophilized to deprotected, polymerized, and purified as described in Compd LIX to obtain 2.3 g (yield 63.62%) of XIV.

Certain physical properties of the above polytricosapeptides, poly(30mers), will be compared with those of the polymers obtained by random polymerization of the composite pentamers, mixed at the desired ratios for the polymerization mixture.

Random Polymerization of Tricosapeptide Six Composite Pentamers

Poly[(GDGFP),2(GVGVP),2(GVGFP),(GFGFP)] (LXV): Compd XXXI (0.8 g, 0.001 mol), Compd XXVIII (1.3 g, 0.002 mol), Compd XXIX (1.4 g, 0.002 mol) and Compd XXX (0.74 g, 0.001 mol) were deprotected together using TFA and a one-molar solution of the TFA salt in DMSO was polymerized for 18 days using 1.6 equivalent of NMM as base. The polymer was dissolved in water, dialyzed using 3500 mol. wt. cut-off tubing, and lyophilized. The side chain was then deprotected using HF: p-cresol (90:10, v/v) at O °C for 1 h. It was triturated with ether and then dissolved in water, base treated using 1 N NaOH, dialyzed using 50,000 mol. wt. cut-off tubing and lyophilized to obtain 2.0 g (yield 73.59%) of LXV.

Poly[(GDGFP),3(GVGVP),2(GFGFP)] (LXVI): Compd XXXI (0.8 g, 0.001 mol), Compd XXVIII (1.95 g, 0.003 mol) and Compd XXX (1.5 g, 0.020 mol) were deblocked together using TFA. The polymerization, deprotection, and purification proceeded as described in Compd LXV to obtain 2.1 g (yield 77.27%) of LXVI.

ism, be it uni- or multi-cellular, becomes the synthesizer.[12-16]

Hyper Expression of the Protein-Based Polymer $(GVGVP)_{121}$ in E. coli

The protein-based polymer gene $(GVGVP)_{121}$ coding for the three amino acids glycine, valine and proline, was inserted into the expression vector pET-11d as described elsewhere.[2,16] The plasmid pET11d (Novagen) contains a T_7 promoter that drives the $(GVGVP)_{121}$ coding sequence, the β-lactamase gene that confers resistance to ampicillin, and the *lac I* gene that produces the repressor that would regulate synthesis of the introduced gene. The pET11d construct containing the $(GVGVP)_{121}$ coding sequence was introduced into the bacterial host strain HMS174 containing a lysogen DE3 in its genomic DNA. DE3 is a lambda derivative that has the immunity region of phage 21 and carries a DNA fragment containing the *lac I* gene, the *lac UV5* promoter and the gene coding for the T_7 polymerase. Therefore, any gene driven by a T_7 promoter introduced into this *E. coli* strain (HMS174 DE3) should be under the control of the repressor protein produced by the *lac I* gene present in the chromosome, as well as in the plasmid, pET11d. The regulation of expression of the introduced foreign gene is at the level of production of the T_7 polymerase: T_7 polymerase will be produced only when the *lac I* gene product, the repressor, is inactivated. This is accomplished by the addition of isopropylthio-β-D-galactoside (IPTG) to the growth medium that enters the bacterial cells, binds to the repressor, and renders the repressor inactive. Lack of binding of the repressor in the operator region of the T_7 polymerase gene initiates transcription and production of the T_7 polymerase, which, in turn, would trigger transcription and synthesis of genes that are driven by T_7 promoters.

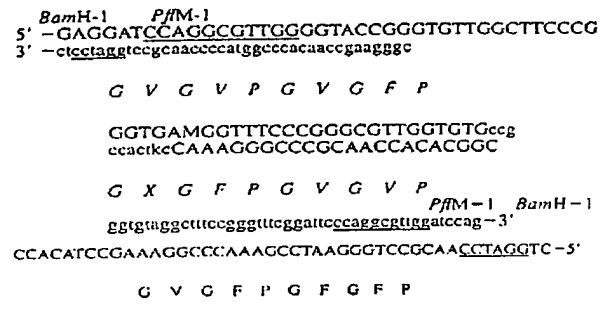
With this background information we present here an overview of the synthesis of $(GVGVP)_{121}$ in *E. coli* strain HMS174(DE3), as described in detail elsewhere.[17] Polymer expression was studied in HMS174 cells grown in Luria broth or Terrific broth for different durations. Cells were grown in the presence of the antibiotic ampicillin and in the presence or absence of IPTG. Light microscopic studies using oil immersion lens showed distinct intracellular inclusion bodies in uninduced cells (grown in the absence of IPTG) but not in induced cells. The first inclusion body generally appeared at one end of the cell. The number and size of inclusion bodies varied with the age and size of individual cells. In order to get more details about the inclusion bodies, cells, grown in the presence of absence of IPTG for different durations, were examined under transmission electron microscope. Cells prefixed in 3% glutaraldehyde in cacodylate buffer were post-fixed in 1% osmium tetroxide; cells solidified in agarose blocks were embedded in epoxy resin, and sections stained with uranyl acetate and lead citrate were observed using a Zeiss transmission electron microscope. Under these conditions, polymer inclusions appeared as glittering bodies amid a dense, dark background of the cell cytoplasm. See Figure 2 showing a transmission electron micrograph of an *E. coli* cell (strain HMS174) transformed with pET-11d containing the $(GVGVP)_{121}$ coding sequence showing polymer production and a control *E. coli* untransformed cell of the same strain. Surprisingly, the inclusion bodies were observed in uninduced cells grown in the absence of IPTG in contrast to current concepts of the operator-promoter functions. Details of these results, including an explanation for the production of polymer in uninduced cells, as well as reasons for hyperexpression of the polymer (at times greater than 80% of the cell volume) are published elsewhere.[17]

Gene Construction and Expression of Polytricosapeptide Genes

Microbial biosynthesis of protein-based polymers requires the use of genetic engineering techniques to construct

FIGURE 2. (A) Non-transformed *E. coli*, and (B) transformed *E. coli* filled with inclusion bodies of $(GVGVP)_{121}$.

synthetic genes and have them expressed inside the microbial cell.[18–20] The genes encoding the polytricosapeptides were constructed in a manner similar to the poly(GVGVP) genes.[2,16] Genes for the tricosapeptides GVGVP GVGFP GEGFP GVGVP GVGFP GFGFP and GVGVP GVGFP GDGFP GVGVP GVGFP GFGFP, analogous to compounds LXII and LIX, respectively, were constructed using synthetic oligonucleotides. The double-stranded DNA sequence of these genes with the corresponding amino acid sequence is the following (Equation 1):



where nucleotides M/k = A/t when amino acid X = E and M/k = C/g when X = D. For each gene, two single-stranded oligonucleotides, indicated by the upper-case letters in the sequence, were annealed through their overlapping regions of complementarity (dashed line) and extended from their 3' ends with DNA polymerase and deoxynucleotide-triphosphates to give the full-length, double-stranded molecule. These genes were then "cloned" by inserting them into the circular plasmid DNA molecule pUC118. This was done by digesting with restriction endonuclease BamH-1 to cleave the gene fragment at each end and to linearize the pUC118 plasmid by a single cut, leaving compatible "cohesive" ends that were joined with DNA ligase enzyme to re-circularize the plasmid containing the gene fragment. The plasmid molecules containing the inserted tricosamer gene fragments were then inserted into cells of *Escherichia coli (E. coli)*; the presence on the plasmid of a gene providing antibiotic resistance to the *E. coli* assures that only those cells that contain the plasmid will be able to grow on media containing the antibiotic. Once inside the cell, the plasmid molecule is amplified by replicating to many copies, or clones, per cell. Growing the cells to a high density in liquid culture provides a source from which large amounts of the plasmid DNA can be extracted. In this case, it was necessary to prepare large amounts of the plasmid as a subsequent source of the basic tricosamer genes needed for building the polytricosamer genes. This was done, and the sequence of the tricosamer genes was verified by DNA sequence analysis on the pUC118 plasmid. The plasmid DNA was then digested with restriction endonuclease PflM-I to release the tricosamer gene fragment. A large amount of the gene fragment was purified and used in a concatenation-ligation reaction to produce gene multimers or "concatemers" encoding a polytricosamer.

Included in the concatenation-ligation reaction were short, double-stranded "adaptor" oligonucleotides. These adaptors provided additional restriction endonuclease recognition sites required for the cloning of the polytricosmer genes into plasmid pUC118 and their subsequent cloning into a variety of expression plasmids. The adaptor oligos were included in the ligation reaction at a ratio that would terminate the concatemer chains resulting in a yield of multimers of the desired length.

EXAMPLE OF A SEQUENCE DEPENDENT PROPERTY, THE pK_a

The greatest advantage of protein-based polymers over all other polymers is the capacity to control sequence. This is combined with the diversity and functional richness of the fundamental repeating unit and is further enhanced by the capacity to introduce enzyme specific sites. The chemical synthesis of even the relatively simple, protein-based polymer, poly(GVGVP), requires great care to obtain the correct properties. The difficulties for chemical synthesis increase many fold as the size and complexity of the repeating unit increases, as, for example, demonstrated above for the polytricosapeptides, poly(30mers).

By means of genetic engineering and expression in *E. coli*, the preparation of the poly(30mer) is not significantly more difficult than that of the poly(5mer), and, once the genetic engineering is achieved, more product is obtained simply by means of an additional fermentation. For chemical synthesis, on the other hand, each synthesis is as taxing as the previous one.

If the sequence of amino acids were not critical, simply mixing amino acids in the correct ratios and polymerizing would be sufficient to obtain polymers of the desired properties, but this is not the case. Chemical synthesis would become easier if mixing pentamers in the correct ratios and polymerizing gave the desired properties, but this is also not the case. As shown below, properties of particular interest, such as an induced pK_a shift, which is a measure of an output of chemical energy, require control of sequence.

Comparison of Chemically Synthesized Polytricosapeptides with Their Random Composite Pentamer Counterparts

In the following, the pK_a values are compared for Asp(D) and Glu(E) residues in poly(30mers) and in polypentapeptides having pentamer compositions similar to those of the polytricosapeptides.

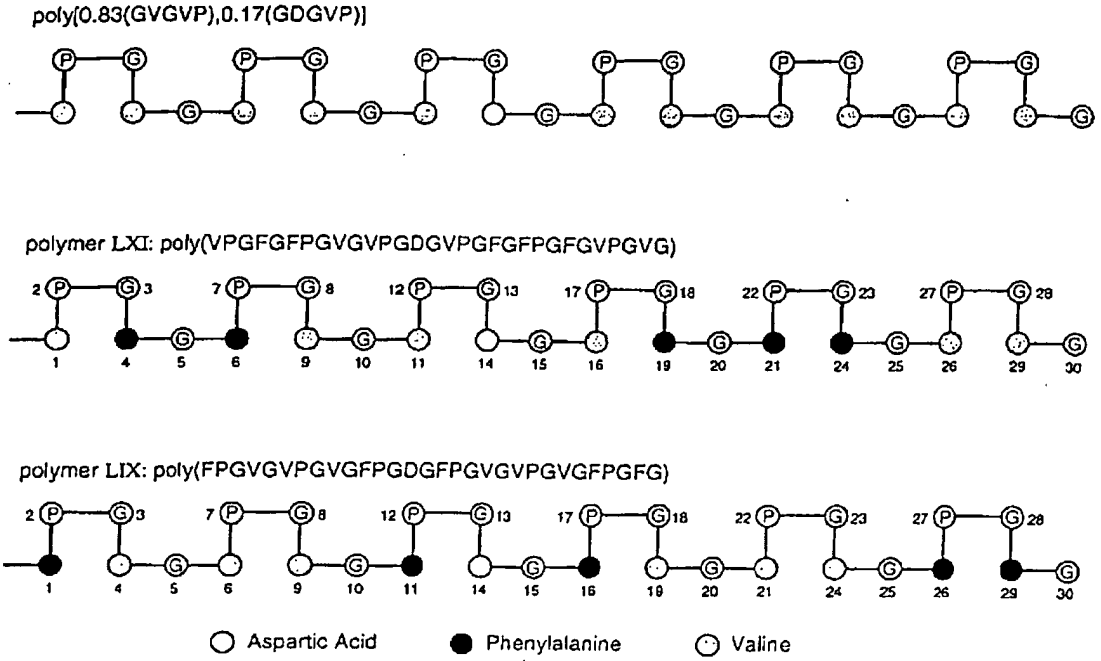


FIGURE 3. Representations of primary structures of poly[0.83(GVGVP),0.17(GDGVP)], polymer LXI and polymer LIX. All polymers have the same mole fraction, 0.17, of pentamers containing the aspartic acid(D) residue. *Source:* Reference 21. Adapted with permission.

pK_a Values for the Aspartic Acid Residue

Figure 3 contains the representation of three primary-structures, each having one aspartic acid(D) residue per thirty residues. Polymers LXI and LIX have exactly the same composition, but differ in sequence, in the relative spatial orientation of the very hydrophobic Phe(F) and chargeable Asp(D) residues, as shown in Figure 4. The experimental pK_a values, determined by means of classical acid-base titrations of Figure 5, differ remarkably.[21] The pKa values are listed in Table I along with the data for the amino acid analyses.

When the composite pentamers of LXI and of LIX were mixed in the desired ratios, polymers LXV and LXVI were obtained. Unfortunately, the pentamers did not incorporate into the polymers in the ratios as mixed. Instead of a mole fraction of 0.17 for the Asp-containing pentamers, the random mix gave values of 0.32 in both cases. Even so, instead of shifting from the normal pK_a of 3.8 or 3.9 for Asp(D) to the extraordinary values near 10, resulting from an optimal primary structure, the random mix of constituent pentamers gave pK_a values near 5.

pK_a Values for the Glutamic Acid Residue

The normal pK_a for Glu(E) is near 4.3, and this is the value for poly[f_V(GVGVP)f_E(GEGVP)] where f_V and f_E are mole fractions with $f_V + f_E = 1$ and with $f_E = 0.17$. As seen in Table I, polymers LXVII and LXVIII exhibit small pKa shifts to values near 5, and they do so with compositions very close to those of the polytricosapeptides, LXII, LXIII, and LXIV. Strikingly, however, the poly(30mers) exhibit pK_a values near 8.[22] Clearly, the achievable pK_a shifts are limited with random incorporation of carboxyl- and Phe-containing pentamers. As the efficient conversion to chemical energy depends to a great extent on the magnitude of the pK_a shift achieved, more efficient conversion becomes possible with protein-based polymers of controlled sequence.

Comparison of Chemically and Microbially Prepared Protein-Based Polymers

The microbial biosynthesis of the above designed Asp(D)− and Glu(E)-containing polytricosapeptides will occur shortly, and it will be of interest to compare their pK_a shifts with those of the chemically synthesized poly(30mers) and to add the effect of varying chain length on the pKa shifts. The property for which comparison is now possible is the chain length dependence of T_t. The set of T_t values for the *E. coli* expressed $(GVGVP)_n$ for n = 41, 141, and 251, were determined from the plots of turbidity versus temperature of Figure 6, and the plot of T_t as a function of log(molecular weight) appears as the inset. The

values of T_t obtained from chemically synthesized polymers using membranes of different molecular weight cut-offs are also included in the inset.[23] When using dialysis membranes for evaluating molecular weight, it should be kept in mind that membrane calibration utilizes globular proteins. Accordingly, it appears that equilibrium dialysis results in an underestimate of the molecular weight, perhaps owing to the filamentous rather than globular nature of these protein-based polymers.

THE ΔT_T HYDROPHOBIC PARADIGM FOR PROTEIN-BASED POLYMER DESIGN

Hydrophobic Folding and Assembly (Inverse Temperature Transitions)

Protein-based polymers can exhibit the property whereby they hydrophobically fold and assemble on raising the

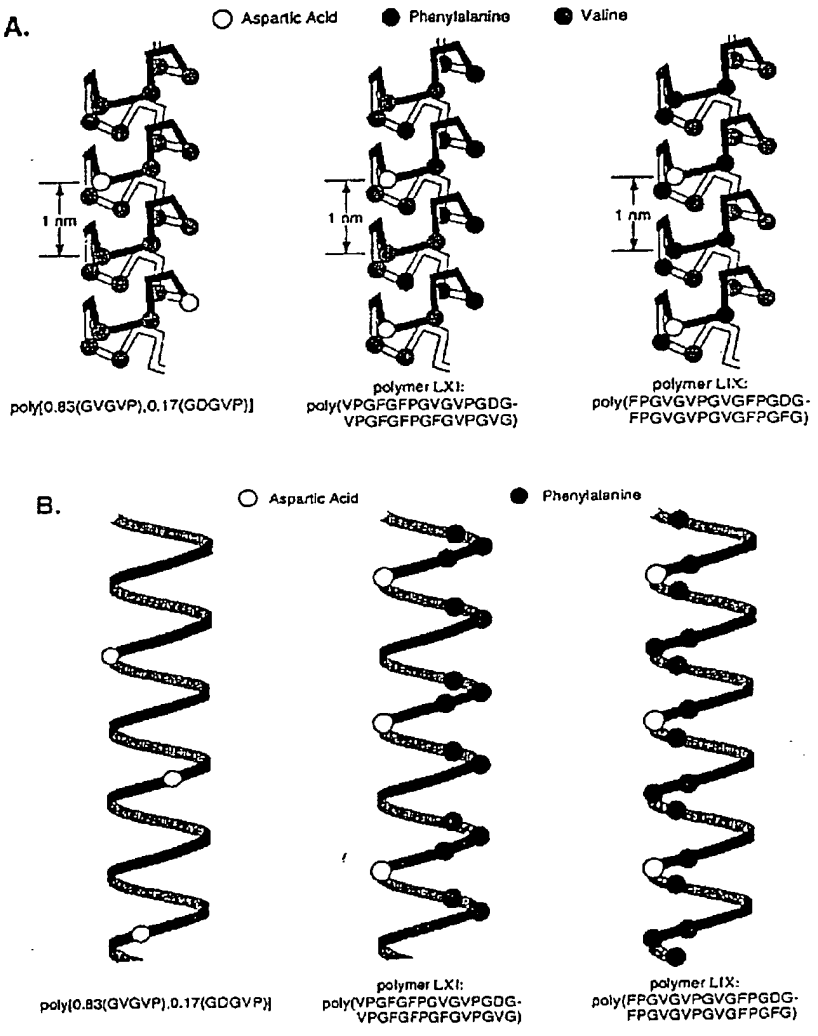


FIGURE 4. Representations of the β-spiral structures, A, and simplified helical structures, B, of poly[0.83(GVGVP),0.17(GDGVP)], polymer LXI and polymer LIX, showing the relative spatial orientations of the Asp(D) and Phe(F) residues in the latter two polymers. In polymer LXI, the hydrophobic Phe(F) residues are distal, and in polymer LIX, the hydrophobic Phe(F) residues are proximal to the Asp(D) residues. *Source:* Reference 21. Adapted with permission.

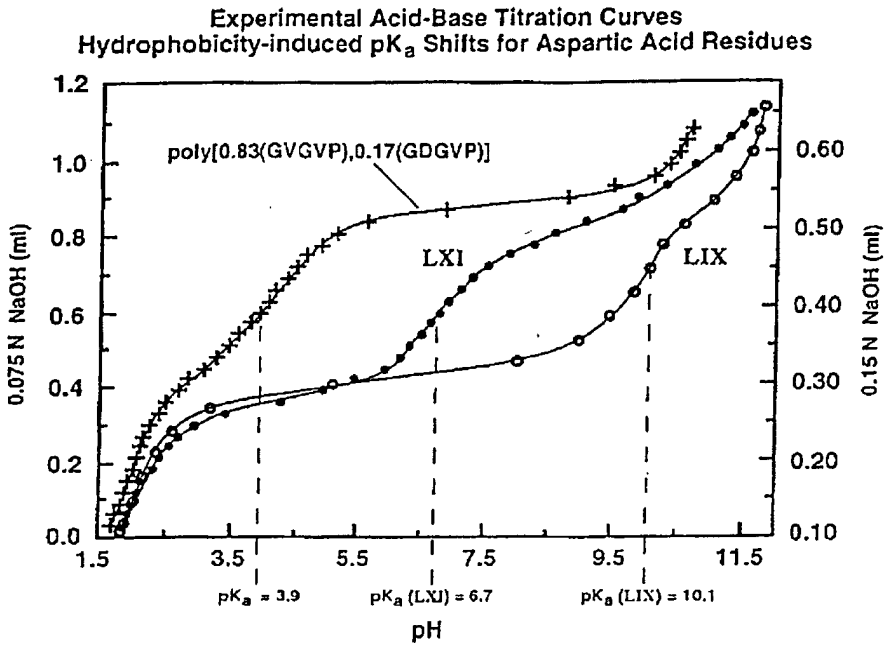


FIGURE 5. Acid base titration curves for poly[0.83(GVGVP),0.17(GDGVP)], with the normal pK_a of 3.9, for polymer LXI with a shifted pKa of 6.7 and for polymer LIX with a dramatically shifted pKa of 10.0. The latter two polymers exhibit much larger pKa shifts than polymers of the same but random composition of pentamers. Control of sequence is essential for large pKa shifts. *Source:* Reference 21. Adapted with permission.

temperature above an onset temperature designated as T_t. This increase in order on increasing the temperature is called an inverse temperature transition. An increase in hydrophobicity, such as the addition of a CH_2 moiety per repeating sequence (as when Val is replaced by Ile), lowers the value of T_t, whereas a decrease in hydrophobicity, such as the removal of two CH_2 moieties per repeat (as when Val is replaced by Ala), raises the value of T_t. Furthermore, any process whereby the value of T_t is lowered from above to below the operating temperature can drive hydrophobic folding and assembly, and any process that causes the value of T_t to be raised from below to above the operating temperature can drive hydrophobic unfolding and disassembly. This principle is called the ΔT_t hydrophobic paradigm of protein-based polymer folding and function.[1,24]

T_t-Based Hydrophobicity Scale for Protein-Based Polymer Engineering

Using polymers of the composition, poly[f_V(GVGVP) f_X(GXGVP)] where f_V and f_X are mole fractions with $f_V + f_X = 1$, and where X is any of the naturally occurring amino acids or an interesting chemical modification thereof, a hydrophobicity scale has been developed based directly on the hydrophobic folding and assembly process of interest. By synthesis of hundreds of such polymers, determining their T_t values and plotting f_X versus T_t, the relative hydrophobicities can be assessed at a convenient value of f_X such as $f_X = 1$. The resulting hydrophobicity scale is given in Table 2.[1,25]

Principles of Energy Conversion by Protein-Based Polymers

These are three underlying principals in protein-based polymer design and function. First, hydrophobic folding and assembly can be controlled by changing the temperature, T_t, at which the hydrophobic folding and assembly transition occurs. This results in related pairs of corollaries. Increasing functional hydrophobicity lowers T_t, and decreasing functional hydrophobicity raises T_t. Consequently, lowering T_t from above to below an operating temperature drives hydrophobic folding and assembly, whereas raising T_t from below to above the operating temperature drives hydrophobic unfolding and disassembly. Second, increasing hydrophobicity, that is, lowering T_t, raises the pK_a of a chemical couple when the charged species occurs at higher pH (for example, COOH/COO−) and lowers the pK_a of a chemical couple when the charged species occurs at lower pH (for example, NH_3^+/NH_2). Third, there are many ways that the

TABLE 1. pK_a Values and Amino Acid Compositions

Polymer Number	Compound	pKa	Asp	Gly	Pro	Val	Phe
LXV	poly[(GDGFP),2(GVGVP),2(GVGFP),(GFGFP)] (random mix of pentamers)	4.6(20°)	0.32±0.02	2.07 ± 0.10	1.0	0.90±0.10	0.70±0.08
LIX	poly[GDGFPGVGVPGVGFPGFGFPGVGVPGVGFP]	10.1(20°)	0.17±0.02	2.02 ± 0.10	1.0	0.92±0.10	0.83±0.08
LXVI	poly[(GDGFP),3(GVGVP),2(GFGFP)] (random mix of pentamers)	5.2(20°)	0.32±0.02	2.05 ± 0.10	1.0	1.01±0.10	0.76±0.08
LX	poly[GDGFPGVGVPGVGVPGVGVPGFGFPGFGFP]	9.5(20°)	0.17±0.02	2.00 ± 0.10	1.0	0.98±0.10	0.82±0.08
LXI	poly[GDGVPGFGFPGFGVPGVGVPGFGFPGGP]	6.7(20°)	0.17±0.02	2.00 ± 0.10	1.0	0.94±0.10	0.85±0.08
	Normal pKa for Asp(D)	3.8(20°)					
		pKa	Glu	Gly	Pro	Val	Phe
LXVII	poly[(GEGFP),2(GVGVP),2(GVGFP),(GFGFP)] (random mix of pentamers)	5.2(20°)	0.15±0.02	1.88 ± 0.10	1.0	1.09±0.10	0.90±0.08
LXII	poly[GEGFPGVGVPGVGFPGFGFPGVGVPGVGFP]	8.1 (20°	0.14±0.02	1.98 ± 0.10	1.0	0.98±0.10	0.81±0.08
LXVIII	poly[(GEGFP),3(GVGVP),2(GFGFP)] (random mix of pentamers)	4.7(20°)	0.15±0.02	2.11 ± 0.10	1.0	1.01±0.10	0.70±0.08
LXIII	poly[GEGFPGVGVPGVGVPGVGVPGFGFPGFGFP]	7.7(20°)	0.16±0.02	2.05 ± 0.10	1.0	0.90±0.10	0.79±0.08
LXIV	poly[GEGVPGFGFPGFGVPGVGVPGFGFPGVGVP]	7.8(20°)	0.18±0.02	1.96 ±.10	1.0	0.93±0.10	0.86±0.08
	Normal pKa for Glu(E)	4.3(20°)					
	Theoretical Values		0.17	2.0	1.0	1.0	0.83

value of T_t, that is the functional hydrophobicity, can be changed for a given polymer composition, chain length, and concentration changing salt concentration, changing concentration of an organic solute, changing polymer side chain ionization, changing polymer phosphorylation, changing pressure, changing the redox state of an attached prosthetic group, changing absorption of light by an attached prosthetic group that is caused by the absorption to undergo a change in hydrophobicity, and changing neutralization of a charged side chain by a counter ion.[1,24,26]

The ΔT_t Mechanism for Performing Mechanical Work

Any energy input that can change the value of T_t for a protein-based can be used to perform mechanical work simply by lowering T_t from above to below the operating temperature to drive hydrophobic folding. This can readily be shown with cross-linked elastic protein-based polymers, those of the composition, poly[f_V(GVGVP)f_X(GXGVP)], as defined above.

Thermo-mechanical Transduction: The effect of 20 Mrads of γ-irradiation on the hydrophobically folded and assembled state of poly[f_V(GVGVP)f_X(GXGVP)] is the formation of an elastomeric band designated as X^{20}-poly(GVGVP), for example. At the temperatures below T_t, the elastic band is swollen. On raising the temperature above T_t, the band will contract with the capacity to lift an attached weight in the performance of mechanical work. This is the conversion of thermal energy into mechanical work, namely, thermo-mechanical transduction.

Chemo-mechanical Transduction: The addition of 1 N NaCl to the solution surrounding X^{20}-poly(GVGVP) lowers T_t by 14 °C. If T_t is 25 °C in the absence of salt and the working temperature is 20 °C, adding a solution of 1N NaCl lowers T_t to 11 °C and drives contraction with the lifting of the weight. This salt-driven performance of mechanical work represents solvent-based, or extrinsic, chemo-mechanical transduction. When the elastomeric band is X^{20}-poly[0.8(GVGVP),0.2(GEGVP)], the band is swollen at pH 7 where the Glu(E) side chain is the carboxylate. On lowering the pH to 2 or 3, the carboxylate is protonated to become COOH; the value of T_t is lowered from 70 °C to 25 °C in phosphate buffered saline, and the band contracts with the performance of mechanical work when the temperature is at an intermediate value of 37 °C. This is polymer-based, or intrinsic, chemo-mechanical transduction.

Electro-mechanical Transduction: When the polymer is poly[0.8(GVGVP),0.2(GK{NMeN}GVP)] where NMeN stands for N-methyl nicotinate attached by amide linkage to the ε-NH_2 of Lys(K), T_t is 49 °C in the oxidized state, and 60% reduction to the N-methyl-1,6-dihydronicotinamide lowers the value of T_t to 9 °C. Accordingly, when at 37 °C, the crosslinked matrix, X^{20}-poly[0.8(GVGVP), 0.2(GK{NMeN}GVP)] is swollen and reduction, either chemically using dithionite or electrochemically using an electron mediator, lowers T_t and drives contraction with the performance of mechanical work. This protein-based polymer has been designed to perform electro-mechanical transduction.

Baro-mechanical Transduction: When the polymer contains aromatic residues, such as Trp(W), Phe(F), or Tyr(Y), the addition of pressure significantly raises the value of T_t because the volume occupied by a water molecule of hydrophobic hydration is less than that of a water molecule in bulk solution. At the appropriate temperature and el-

evated pressure, X^{20}-poly[0.75(GVGVP),0.25(GFGVP)] will be swollen. As the pressure is released, this elastomeric band contracts with the performance of mechanical work. This is baro-mechanical transduction.

Photo-mechanical Transduction: When the polymer is poly[0.68(GVGVP),0.32(GE{AB}GVP)] where {AB} stands for azobenzene attached to the side chain of Glu(E) by amide linkage, irradiation with 350 nm light at pH 4.1 in phosphate buffered saline causes the value of T_t to increase from 32° to 42 °C when the substitution is about 50%.[27] The absorption of 350 nm light by azobenzene causes the chromophore to convert from the *trans* to the less polar, more hydrophobic *cis* geometrical isomer. Similarly, when the cross-linked elastic matrix is X^{20}-poly[0.8(GVGVP), 0.2(GK{CA}GVP)] where {CA} stands for cinnamic acid attached by amide linkage to the ε-NH_2 of Lys(K), irradiating with 300 nm light converts the chromophore from *trans* to *cis*, raises the value of T_t, and causes the matrix to swell.[28] This is photo-mechanical transduction.

The $\Delta T_t/\Delta pK_a$-Mechanism for Performing Chemical Work

The Relationship between ΔT_t and ΔpK_a: As seen in Table 2, increasing the number of Phe(F) residues in a polymer lowers the value of T_t, and as seen in Table 1, increasing the number of the carboxylic acid moieties of the Glu and Asp residues. Therefore, lowering the value of T_t raises the pKa of the carboxylic acid side chains of the Glu(E) and Asp(D) residues. This is one of numerous examples whereby increasing hydrophobicity lowers the value of T_t and raises the pK_a value of the carboxyl/carboxylate chemical couple.[21,22,29-31]

The Relationship between ΔpK_a and ΔE: The change in chemical energy, ΔE, is $\Delta\mu \times \Delta n$ where μ is the chemical potential and n is the number of moles of species undergoing a change. The chemical potential is defined as $\mu = RT \ln a$ where a is the activity of the chemical species. At the low proton concentrations, $[H^+]$, of interest here, concentration and activity are equivalent such that $\mu = RT \ln[H^+]$. As it is common to work in logarithms to the base ten, $\mu = 2.3\ RT \log[H^+]$, but since $pH = -\log [H^+]$, $\mu = -2.3\ RT\ pH$. Accordingly, the change in proton chemical potential required to keep a polymer at 50% ionization, that is, at its pK_a, becomes $\Delta\mu = -2.3\ RT\ \Delta pKa$. Therefore, for a Δn of 1, the ΔpK_a is a measure of the change in chemical energy, ΔE, and any energy input that changes T_t and thereby changes the pK_a, is capable of performing chemical work.

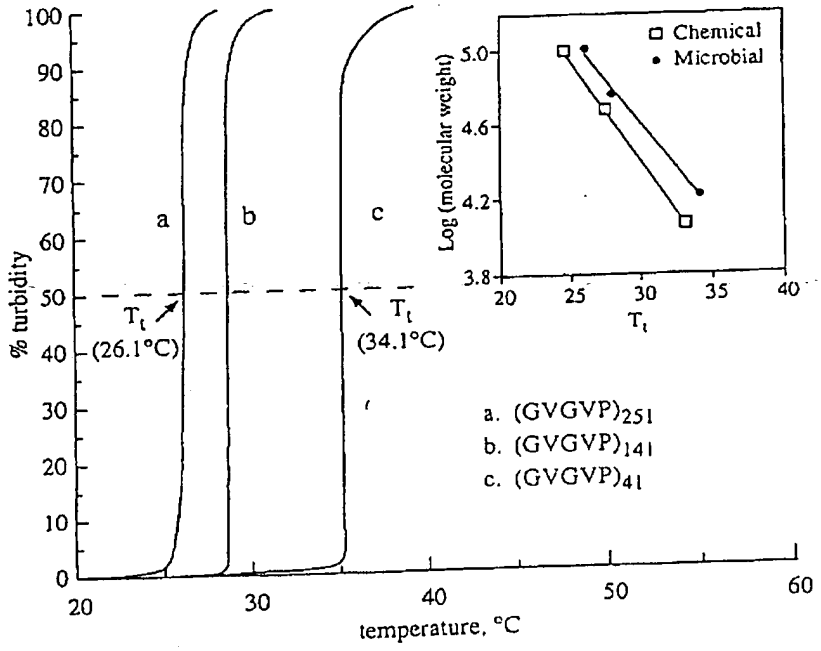


FIGURE 6. Temperature profiles for the inverse temperature transitions of three microbial preparations of $(GVGVP)_n$ with N = 41, 141, and 251. These values are plotted in the inset as log (molecular weight) versus T_t and compared to chemically synthesized poly(GVGVP), which were fractionated according to size by membrane dialysis.

TABLE 2. T_t-Based Hydrophobicity Scale for Proteins T_t=Temperature of Inverse Temperature Transition for poly[f_v(VPGVG), f_X(VPGXG)]

Residue X		T_t, linearly extrapolated to $f_X = 1$	Correlation Coefficient
Lys(NMeN, reduced)[a]		−130 °C	1.000
Trp	(W)	−90 °C	0.993
Tyr	(Y)	−55 °C	0.999
Phe	(F)	−30 °C	0.999
His (pH 8)	(H°)	−10 °C	1.000
Pro	(P)[b]	(−8 °C)	calculated
Leu	(L)	5 °C	0.999
Ile	(I)	10 °C	0.999
Met	(M)	20 °C	0.996
Val	(V)	24 °C	reference
Glu($COOCH_3$)	(E^m)	25 °C	1.000
Glu(COOH)	(E^o)	30 °C	1.000
Cys	(C)	30 °C	1.000
His (pH 4)	(H^+)	30 °C	1.000
Lys(NH_2)	(K^o)	35 °C	0.936
Pro	(P)[c]	40 °C	0.950
Asp(COOH)	(D^o)	45 °C	0.994
Ala	(A)	45 °C	0.997
HyP		50 °C	0.998
Asn	(N)	50 °C	0.997
Ser	(S)	50 °C	0.997
Thr	(T)	50 °C	0.999
Gly	(G)	55 °C	0.999
Arg	(R)	60 °C	1.000
Gln	(Q)	60 °C	0.999
Lys(NH_3^+)	(K^+)	120 °C	0.999
Tyr(ϕ-O^-)	(Y^-)	120 °C	0.996
Lys(NMeN, oxidized)[a]		120 °C	1.000
Asp(COO^-)	(D^-)	170 °C	0.999
Glu(COO^-)	(E^-)	250 °C	1.000
Ser(PO_3^-)		1000 °C	1.000

[a]NMeN is for *N*-methyl nicotinamide pendant on a lysyl side chain, that is, N-Methyl nicotinate attached by amide linkage to the ε-NH_2 of Lys and the reduced state is *N*-methyl-1,6-dihydronicotinamide.
[b]The calculated T_t value for Pro comes from poly(VPGVG) when the experimental values of Val and Gly are used. This hydrophobicity value of −8 °C is unique to the β-spiral structure where there is hydrophobic contact between the $Val^1\gamma CH_3$ and $Pro^2\beta CH_2$ moieties.
[c]The experimental value determined from poly[f_V(VPGVG), f_P(PPGVG)].
Source: Reference 23. Adapted with permission.

Means of Performing Chemical Work: With the proper polymer design, each of the above energy inputs that resulted in a change in T_t with the performance of mechanical work should be able to perform chemical work. The protein-based polymer design required to convert various energy inputs to chemical energy can utilize a charged (polar)/uncharged(apolar) chemical couple as the actuator and, as the sensor, can use a chemical entity that changes its polarity (hydrophobicity) in response to a particular energy input. Thus a pair of functional groups are used that are coupled by virtue of being part of the same hydrophobic folding domain.

In principle, each of the following can perform chemical work when part of a properly designed protein-based polymer: changing the oxidative state of an attached redox couple (electro-chemical transduction), absorption of light by an attached chromophore that changes its hydrophobicity as when going from *trans* to *cis* (photo-chemical transduction), and the decrease in volume as waters of hydration form around aromatic side chains in response to an applied pressure (baro-chemical transduction). It is also possible to design a polymer capable of various forms of chemo-chemical transduction. In addition, the application of mechanical force to stretch, for example, X^{20}-poly [0.8(GVGVP),0.2(GEGVP)], causes an increase in pK_a.[32] Therefore, the input of mechanical energy results in the performance of chemical work of proton uptake (that is, mechano-chemical transduction).[32,33]

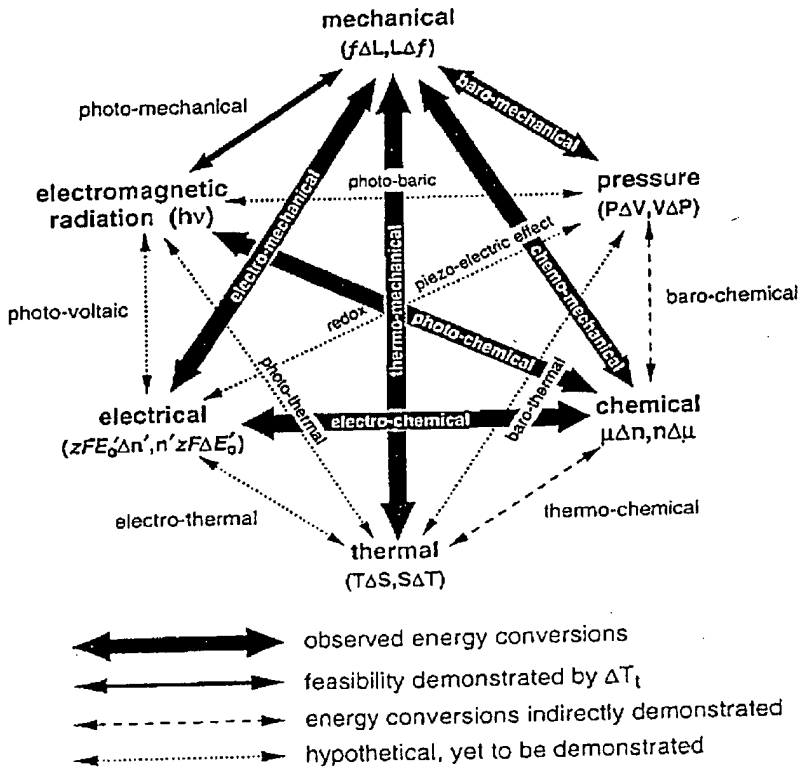


FIGURE 7. Demonstrated and putative pairwise energy conversions using the ΔT_t mechanism. *Source:* Reference 1. Adapted with permission.

All of the demonstrated and putative energy conversions of which protein-based polymers are considered capable of performing are shown in Figure 7.[1] It appears possible to design a protein-based polymer to sense any of six particular energy changes and to actuate, that is to output, in any of the six forms of the free energy, the intensive variables of which are mechanical force, temperature, pressure, chemical potential, electrochemical potential and light (that is, electromagnetic radiation). The potential and versatility of protein-based polymers can be seen by applications as far-ranging as the prevention of adhesions, tissue reconstruction, controlled delivery of therapeutic substances, and agricultural enhancement factors and biodegradable plastics. The future of protein-based polymers looks promising and exciting.

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Proteins

See: Biopolymers (Overview)
Biopolymers (Electrochemical Behavior)
Cellulose Derivative-Protein Complex (in Ice Cream)
Cellulose Derivative-Protein Complexes (Whey and Corn Steep Liquor. Enrichment of Macaroni)
Cellulose Derivatives (Reclaimation of Proteins from Cheese Whey)
Collagen
Connectin (Titin) (Large Filamentous Protein)
Gelatin (Molecular Weight Distributions)
Globular Proteins (Structures and Functions)
Gluten Plastic, Biodegradable
Hemoproteins
Keratins
Marine Adhesive Proteins, Synthetic
Monolayer and Langmuir-Blodgett Films (of Protein)
Nonthrombogenic Polymers (Multiblock Copolymers of Polyether and Polyamide)
Nucleohistone Complexes (Protein-Nucleic Acid Interactions)
Poly(α-amino acid) Spherical Particles
Protein-Based Polymeric Materials (Synthesis and Properties)
Radiation Induced Polymerization (Biomedical Applications)
Reverse Micelles (Microcontainers for Functinal Polymers)
Self-Assembled Polymers (at Interfaces)
Silk (Its Formation, Structure, Character, and Utilization)
Silk (Physico-Chemical Properties)
Silk Fibers (Chemical Modification)
Silk Fibers (Grafting)
Silk Fibroin (Soft Tissue Compatible Polymer)
Spider Silk (Production of Polypeptide Polymers)
Textile Fibers (Structure and Properties)
Wool (Overview)
Wool Grafting
Wool Keratin

Pseudo-Poly(amino acid)s

See: Controlled Drug Delivery Systems
Pseudo-Poly(Amino Acid)s (Overview)

PSEUDO-POLY(AMINO ACID)S (OVERVIEW)

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Pseudo-poly(amino acid)s are derived from naturally occurring amino acids. Unlike conventional poly(amino

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Plant Cell Reports

Somatic embryogenesis and plant regeneration in cotton (*Gossypium hirsutum* L.)

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Abstract

Tissue culture methods for improvement of cotton has lagged seriously compared to other major crops. A method for regeneration of cotton which includes a morphogenetically competent cell suspension was needed to facilitate selection of stress-resistant variants and gene manipulation. Preliminary screening of eight strains of Gossypium hirsutum L. for embryogenic potential resulted in the production of somatic embryos in all strains. Coker 312 was selected for use in the development of a model regeneration system for G. hirsutum. Calli were initiated from hypocotyl tissues of 3-day-old-seedlings. Globular embryos were present after six weeks in culture. Calli were subcultured to liquid suspension in growth regulator-free medium. After three to four weeks, suspensions were sieved to collect globular and heart stage embryos. Collected embryos developed further when plated onto semi-solid medium. To induce germination and plantlet growth, mature embryos were placed on sterile vermiculite saturated with medium. Upon development of roots and two true leaves, plantlets were potted in peat and sand, and hardened. Mature plants and progeny have been obtained with this procedure. A high percentage of infertile plants was observed among the regenerants.

Key Words: Gossypium hirsutum – somatic embryogenesis.

Abbreviations: 1 naphthaleneacetic acid (NAA), indole-3-acetic acid (IAA), 2,4-dichlorophenoxyacetic acid (2,4-D), gibberellic acid (GA_3), Murashige and Skoog (MS), 6 benzylamino purine (BA), N^6-(^{2}-isopentenyladenine (2i P).

Introduction

Somatic embryogenesis and subsequent plant regeneration has been reported in most of the major crop species (Evans et. al., 1981) with soybean (Glycine max), and cotton (Gossypium) proving to be the most difficult to regenerate (Scowcroft, 1984). Price and Smith (1979) first reported embryogenesis in Gossypium klotzschianum Anderss but plants were not regenerated. Progress in the regeneration of Gossypium species has not been achieved rapidly, with the first report of regeneration of Gossypium hirsutum by Davidonis and Hamilton (1983). Because of the lengthy culture period, this method was undesirable and not easily repeated. Shoemaker et al. (1986) recently described somatic embryogenesis and plant regeneration from G. hirsutum var Coker 315 and 201 in which calli were selected and subcultured to produce a few mature embryos each transfer. A comprehensive procedure for regeneration which includes a morphogenetically competent cell suspension has yet to be developed. A protocol for consistently regenerating cotton is needed so that selection of stress-resistant variants and gene manipulation can be made feasible as methods for improving existing breeding lines. The objective of this study was to develop and evaluate a procedure which could be used as a standard for regeneration of cotton.

Materials and Methods

Seed of Gossypium hirsutum L. var Coker 312 and 5110 were obtained from Coker Seed Inc. and T25 and T169 (Texas collection), Paymaster 303 and 784, and RQSX-1-1 (G. hirsutum x G. barbadense hybrid) and Stoneville 213 from Dr. Jerry Quisenberry USDA, ARS, Lubbock, Texas.

Callus Initiation and Maintenance

Seed were sterilized by dipping in 70% ethanol prior to a 20 minute exposure to 10% Clorox containing one drop of Tween 20 per 100 ml, rinsed in sterile distilled water, and germinated on sterile germination blotters in petri dishes under 30 µE $m^{-2}s^{-1}$ light and 28 ± 2°C. Three days after emergence of the radicle, the hypocotyl was sectioned into 4 mm lengths. The medium used for callus induction was that of Murashige and Skoog (1962) supplemented with 100 mg/l myo-inositol, B5 vitamins (Gamborg, 1968) and 30 g/l glucose. The medium was solidified with 1.6 g/l Gelrite, and 0.75 g/l $MgCl_2$. The pH of the medium was adjusted to 5.8 prior to autoclaving for 20 minutes at 121°C. Four growth regulator regimes were evaluated in the initial screening of genotypes: 0.05 mg/l 2,4-D, 0.1 mg/l 2,4-D, 0.1 mg/l 2,4-D + 0.1 mg/l kinetin, and 0.1 mg/l 2,4-D + 0.5 mg/l kinetin. All growth regulators were added to the medium prior to autoclaving. For the genotype screen, two hypocotyl sections from each of two seedlings of each genotype were placed on each of the four callus induction media on three dates. Explants were incubated in 25 x 150 mm culture tubes under a 16:8 hour photoperiod at 60 to 90 µEm $^{-2}s^{-1}$ and 28 ± 2°C (cool white and full spectrum fluorescent lights).

Calli were evaluated 30 days after initiation and

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again after 6 weeks culture. Callus was dispersed in sterile water and examined with a stereo-microscope for globular and heart stage embryos comparable to those observed in vivo. The basis of selection of a genotype for further studies was the total number of embryos observed in the samples examined and their comparability to in vivo embryos at similar stages of development. Coker 312 was selected for further evaluation. In all subsequent experiments for development of a model system, hypocotyls of Coker 312 were cultured as previously described except that only the growth regulator regimes containing kinetin were used. Calli obtained were subcultured after four weeks to the same medium or transferred to cell suspension for further experimentation.

Cell Suspension Initiation and Maintenance

Calli of Coker 312 were transferred from the initiation medium to cell suspension after one month. Calli have also been subcultured on the initiation medium for up to one year prior to liquid culture. Medium for liquid culture was the same as callus initiation medium, but was devoid of growth regulators and gelling agents (basal medium). Approximately 100 mg of callus tissue per 10 ml of liquid medium was used in the initial inoculation. After three to four weeks, the suspension was sieved with 1 layer of cheesecloth, or 10 mesh Cellector (EC Apparatus), or soil sieve. The filtrate was then collected on 2 layers of cheesecloth or 60 mesh sieve. The resulting mass was washed twice with basal medium and resuspended at not less than 40 mg/ml wet weight. Liquid cultures were rotated at 120 rpm under the same light and temperature regimes previously described. Embryogenic cell suspensions were maintained by this routine every three to six weeks. Time of subculture and precise density was dependent upon the particular cell line.

Plating of Cell Suspensions

Development of embryos was carried out on semi-solid medium. Embryogenic suspensions were sieved three to four weeks after subculture. Three fractions were obtained by sieving the suspensions with 10, 20, and 30 mesh screens. Mesh size 10 collected larger tissue clumps, embryos and embryo aggregates (fraction I). Mesh size 20 collected large globular and heart stage embryos and small embryo aggregates (fraction II). Fraction III was obtained with a 30 mesh sieve and contained single cells, pro-embryonic units, and small globular and heart stage embryos. Pro-embryonic units are defined as asymmetric clumps of extremely small cells, having an epidermal like surface, but which cannot be distinguished as globular embryos. Fractions II and III were resuspended in basal medium at a minimum of 40 mg/ml and two ml then pipetted onto the surface of 25 x 100 mm petri plates containing 40 ml of basal medium with an additional 1.9 g/l KNO_3, 1.6 g/l Gelrite, and 0.75 g/l $MgCl_2$. Plates were incubated at 28 $\pm$ 2°C and 90 µE m^{-2} s^{-1}.

Fraction II required four weeks and fraction III, six weeks, for embryos to reach 3 to 10 mm in size. Embryo proliferation and embryo maturation occurred during this time. Embryogenic masses of approximately 400 mg were selected from each plate and subcultured to the same medium at four to six week intervals. Embryos $\geq$ 3 mm in size were selected for germination.

Embryo Germination and Plant Regeneration

Mature ($\geq$ 10 mm in size), nearly mature ($\geq$ 5 mm < 10 mm) and immature ($\geq$ 3 mm < 5 mm) embryos obtained from plated cultures were placed in 25 x 150 mm culture tubes containing one culture tube cap (Kaput) of #2 Vermiculite saturated with 10 to 12 ml of Stewart and Hsu's medium (1977) containing 0.1 mg/l IAA. Embryos were incubated at 28 $\pm$ 2°C and 90 µE m^{-2} s^{-1}. During the germination and shoot formation stages, fresh medium was added to the vermiculite when liquid was no longer visible at the bottom of the culture tube. Plants which developed an extensive root system and at least two true leaves were potted in 15 cm plastic containers in a one-to-one mix of peat and sand and watered with 1/4 strength Hoagland's (1936) solution. A 10 ml (dependent on plant size) glass beaker was placed over the plant for three days; followed by 30 ml, 50 ml, 100 ml, and 150 ml beakers sequentially exchanged every three days thereafter. Three days after removal of the 150 ml beaker, plants were placed in the greenhouse.

Results and Discussion

Callus Initiation and Genotype Screen

Coker 312 produced embryos under all growth regulator regimes (Table 1). When embryos were compared to in vivo embryos, Coker 312 globular and heart stage embryos differed from zygotic embryos only by their larger size. T25, while given equal weight in terms of embryo normality, failed to produce embryos at the lowest growth regulator regime. T169 also failed to produce embryos at this lowest level and embryos were not as comparable to in vivo embryos. A clearly defined epidermal surface was lacking. We have considered this neomorphic. Based on this preliminary data Coker 312 was chosen to develop a model regeneration system. G. hirsutum Coker varieties appear to be superior to other varieties in their embryogenic response (Shoemaker et al. 1986; Davidonis and Hamilton 1983).

Table 1. Embryogenic potential of cultivars as indicated by total number of embryos observed.*

	Growth Regulator (mg/l)			
Cultivar	0.5 2,4-D	0.1 2,4-D	0.1 2,4-D 0.1 kinetin	0.1 2,4-D 0.5 kinetin
C312	20	10	14	2
C5110	0	5	0	0
T25	0	50	21	15
T169	0	34	20	134
P303	0	9	0	0
P784	0	12	0	0
ST213	0	9	4	0
RQSX-1-1	7	0	20	55

*100 mg tissue samples of each culture from three induction periods with two replicates per period were evaluated over 42 days in culture.

Induction of somatic embryogenesis in Coker 312 was repeated with the cytokinin containing media. Hypocotyl segments of 10 three-day-old seedlings (3 segments per seedling) were cultured as described in materials and methods. Callus was allowed to grow without subculture for 60 days prior to evaluation. Embryogenesis occured during the callus initiation stage without need for a second induction medium. Embryogenic calli in these media were friable and light grey green in color. The 0.5 mg/l kinetin medium resulted in a higher percentage of embryogenic calli (Table 2), but tissues became brown at an earlier time, and callus friability was less than when 0.1 mg/l kinetin was employed. The growth regulator regime subsequently used as a standard for induction of somatic embryogenesis was 0.1 mg/l 2,4-D + 0.1 mg/l kinetin.

Table 2. Fresh weight (FW) and percent of Gossypium hirsutum cv Coker 312 calli which form somatic embryos (%E) with 0.1 mg/l 2,4-D and the kinetin concentration listed. Explants were hypocotyl of 3-day-old seedlings.

	Kinetin (mg/l)			
	0.1		0.5	
	%E	fw(g)	%E	fw(g)
seedlings (n = 10)	90.0		100	
explants (n = 30)	46.7	5.7 ± 0.5	96.7	6.1 ± 1.3

Cell Suspension

Calli were initiated with the 0.1 mg/l 2,4-D + 0.1 mg/l kinetin growth regulator regime. Calli maintained for 1 month to 1 year were transferred to cell suspension. One month old callus rarely contained embryos when transferred whereas 6 weeks old callus frequently contained numerous globular embryos. Calli transferred to cell suspension one month after induction often was not embryogenic until the second subculture in basal medium.

The optimum density of cells for subculture varied between cell lines. Two cell lines were subcultured at densities of 12 mg/ml, 50 mg/ml, 150 mg/ml and 200 mg/ml wet weight with four replicates each of fraction I and fractions II and III (combined) per density level per cell line. Subcultures of cell line 1 grew more rapidly than cell line 2. All fraction I cultures proliferated more rapidly than II/III but produced high levels of phenolics and embryos rarely advanced beyond the globular stage whereas embryos reached the torpedo stage in II/III. At the two highest fraction II/III densities, embryo proliferation predominated in cell line 1 and few torpedo stage embryos developed. Density effects may be similar to those observed by Sung and Okimoto (1983) in carrot cell lines whereby high density, phytohormones, and/or conditioned medium allowed proliferation but not further development. These observations indicate that the inocula fraction and density used in this study is not necessarily the optimum at which a Coker 312 cell suspension will perform well. When the optimum density and subculture routine was established for a cell line, the culture could be maintained for one year without apparent decline in embryogenic potential.

The embryogenic competence of cell suspension and plated cultures was maintained without periodic exposure to phytohormones. It has been our experience that cotton cell cultures from several genotypes become habituated. This suggests that periodic exposure to phytohormones to maintain competence of Coker 312 cultures may be unnecessary because of endogenously produced phytohormones. High levels of endogenously auxin in habituated embryogenic cultures of Citrus sinesis have been implicated in the gradual decline of embryogenic potential of longterm cultures. Treatment with auxin inhibitors improved differentiation (Kochba and Button, 1974; Kochba and Speigel-Roy, 1977, a,b). It is yet to be determined if a longer period of continuous culture of Gossypium hirsutum cv Coker 312 will exhibit a similar decline in embryogenic potential. Additionally, Lupotto (1983) has described recurrent somatic embryogenesis in Medicago sativa L. in which embryo proliferation by secondary adventive embryos did not require periodic exposure to phytohormones.

Plating of Cell Suspension

Mature embryos were obtained within four to six weeks after plating. In the initial plating 24 ± 2.31 embryos (≥ 3 mm in size) per plate were obtained from approximately 80 mg of embryogenic suspension. The number of embryos obtained from subsequent subculture of individual plates has been more variable. Embryogenic areas selected for subculture, which are of equal mass, may not have equal embryogenic potential. Size of the embryos within the mass transferred, friability, and browning, as well as amount of tissue, may affect development. Transfer of greater than 400 mg/plate of embryogenic material often led to rapid proliferation, reversion to callus, and/or failure of globular embryos to mature.

Table 3 illustrates the variability of one initial plate that was subcultured for 24 weeks. Callus was maintained as described for 18 weeks prior to cell suspension. The cell suspension was maintained for 12 weeks prior to plating. Approximately 400 mg of the embryogenic mass of each plate was subcultured at 4 to 6 week intervals. Analysis of variance revealed no significant differences among plates or subcultures. Estimation of variance components indicated that 97.3% of the observed variation is attributed to within plate variance. One probable cause of this variation is subjective selection of embryogenic material during subculture; however, such selection of embryogenic material is critical to maintenance of the cell line. Criteria for selection should be studied to improve variability. Equally important for maintenance is subculture timing. Plated cultures were maintained by routine subculture to the same medium every four to five weeks. If cultures were neglected, globular embryos became a translucent white, older embryos and medium became yellowish, and transfer to fresh medium did not always stimulate embryo development and proliferation. With careful selection and attention to subculture timing, some cultures remained embryogenic for two years. Contrary to Shoemaker et al. (1986) we did not find a requirement for sucrose or hormones for continued embryogenicity. This departure might be attributed to the initial induction medium, a carbohydrate hormone interaction, genotype, or phytohormone habituation as previously discussed.

Table 3. Number of Coker 312 embryos ≥ 3 mm in size obtained over four 4-6 week subculture periods. All plates were subcultured from one initial plate.

Plate No.	Subculture No.				Mean
	1	2	3	4	
1	18	28	16	12	18.5 ± 6.8
2	15	16	23	37	22.8 ± 10.1
3	5	24	23	18	17.5 ± 8.7
4	42	11	23	43	29.8 ± 15.5
5	30	14	9	8	15.2 ± 10.2
6	18	1	14	29	15.5 ± 11.6
7	9	14	17	6	11.5 ± 4.9
8	12	4	2	6	6.0 ± 4.3
9	21	19	0	9	12.2 ± 9.7
10	2	38	10	0	12.5 ± 17.5
Mean	±7.2 −11.9	±6.9 −11.0	±3.7 −8.4	±6.8 −14.6	

All stages of the normal zygotic sequence of embryogenesis were seen in the plated cultures (Fig. 1). However, not all embryos went through the normal sequence. This appeared to be due in part to the stage of development of the embryo when moved to fresh medium. Globular embryos greater than 1 mm in size tended to become enlarged and vitreous when transferred, whereas smaller embryos more often completed the normal stages of embryo development.

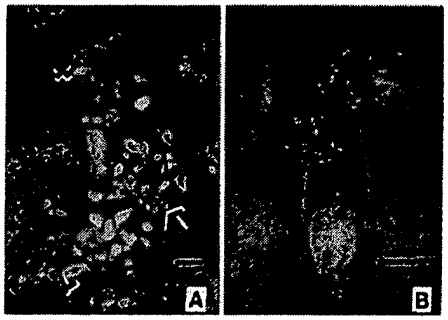

Figure 1. a) Embryogenic callus of Gossypium hirsutum L. cv Coker 312 with various stages of somatic embryo development present: † - globular, Y - torpedo, + - mature. Size bar = 0.5 cm. b) Mature somatic embryo. Size bar = 0.2 cm.

Expansion of cotyledons was variable and the size of the embryo axis appeared more critical to germination and plant growth than presence or size of cotyledons. When embryos $\geq$3 mm were placed on vermiculite for germination, 10.6% of the embryos germinated, developed an extensive root system and produced two or more true leaves within a three month period. Addition of fresh Stewart and Hsu's medium containing 0.1 mg/l GA_3 stimulated plant development even after embryos had been unresponsive for six months.

When $\geq$5 mm embryos were transferred to vermiculite, plant recovery increased to 30% and some embryos without cotyledons produced plants. Embryos < 5 mm were washed with sterile water and placed in liquid medium just covering the bottom of a petri dish for one week. The medium was the same composition as the plating medium with the addition of 0.1 mg/l IAA. Embryos were then washed and placed in fresh medium for a second or a third time period. Embryos that were initially 2 to 3 mm reached sizes of up to 10 mm. These were placed on vermiculite with Stewart and Hsu's medium + 0.1 mg/l GA_3 and 30% recovery obtained within six weeks.

Over a one year period 139 plants were regenerated. Sixty to eighty percent of any one group of regenerated plants hardened successfully. Subsequently, some plants succumbed within the first two months of growth. All remaining plants flowered (78), although, only 15.4% set seed (Fig. 2). Pollen viability of most of the infertile plants was 0%. It is likely that length of time in culture was a factor. One cell line accounted for 41% of the infertile plants. This cell line had been subcultured for 1 year as callus prior to cell suspension and plating. All plants regenerated from this line were infertile.

Other reports of regeneration of cotton (Davidonis and Hamilton, 1983; Shoemaker et al. 1986) have not addressed normality of regenerated plants. The large number of infertile plants reported in this paper indicates that future studies should include the effects of length of culture time and phytohormones on the normality of regenerated plants.

This system is simple, easy to manipulate and can provide large numbers of embryos for study in a short period of time. The establishment of a continuous embryogenic cell suspension enhances the research potential of somatic embryogenesis in cotton.

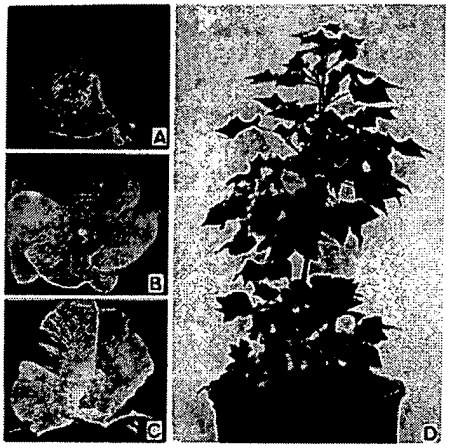

Figure 2. Flowers of Gossypium hirsutum L. Coker 312 a. Normal flower from regenerated plant, b. Abnormal flower from regenerated plant with elongated pistil and reduced number of anthers, c. Normal flowers from normal plant, d. regenerated C312 plant.

Acknowledgment

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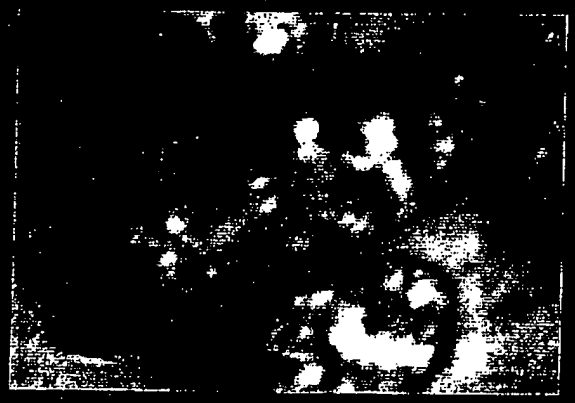
RECOMBINANT PROTEIN PROTOCOLS

Detection and Isolation

Edited by

Rocky S. Tuan



Humana Press

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Hyperexpression of a Synthetic Protein-Based Polymer Gene

Henry Daniell, Chittibabu Guda, David T. McPherson, Xiaorong Zhang, Jie Xu, and Dan W. Urry

1. Introduction

Environmental problems require the development of biodegradable plastics of benign production that can be synthesized from renewable resources without the use of toxic and hazardous chemicals and will help in solving the increasing global disposal burden. Protein-based polymers offer a wide range of materials similar to that of petroleum-based polymers such as elastomers and plastics. Protein-based polymers can be prepared of varied design and composition through genetic engineering without the use of hazardous and noxious solvents and can be made biodegradable with chemical clocks to set their half-lives such that they can be environmentally friendly over their complete life cycles of production and disposal. Compositions tested to date have been shown to be extraordinarily biocompatible, allowing for medical applications ranging from the prevention of postsurgical adhesions and tissue reconstruction to programmed drug delivery *(1)*. Among nonmedical applications, there are transducers, molecular machines, super absorbents, biodegradable plastics, and controlled release of agricultural crop enhancement agents like herbicides, pesticides, and growth factors.

Bioelastic materials are based on elastomeric and related polypeptides comprised of repeating peptide sequences *(2)*; they may also be called elastic and plastic protein-based polymers. The parent polymer, $(Val^1\text{-}Pro^2\text{-}Gly^3\text{-}Val^4\text{-}Gly^5)_n$ or poly(VPGVG), derives from sequences that occur in all sequenced mammalian elastin proteins *(3)*. In the most striking example, the sequence $(VPGVG)_n$ occurs in bovine elastin with $n = 11$ without a single substitution *(3)*. A particularly interesting analog is poly (AVGVP) as it reversibly forms a plastic on raising the temperature.

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What is required for the commercial viability of protein-based polymers is a cost of production that would begin to rival that of petroleum-based polymers. The potential to do so resides in low cost bioproduction. We have recently demonstrated a dramatic hyperexpression of an elastin protein-based polymer, $(Gly\text{-}Val\text{-}Gly\text{-}Val\text{-}Pro)_n$ or poly(GVGVP), which is a parent polymer for a diverse set of polymers that exhibit inverse temperature transitions of hydrophobic folding, and assembly as the temperature is raised through a transition range and which can exist in hydrogel, elastic, and plastic states. Electron micrographs revealed formation of inclusion bodies in *E. coli* cells occupying up to 80–90% of the cell volume under optimal growth conditions *(3a)*. The beauty of this approach is the lack of any need for extraneous sequences for the purposes of purification *(4)* or adequate expression. The usual strategy for expression of a foreign protein or protein-based polymer in an organism such as *E. coli* anticipates that the foreign protein will be injurious to the organism. Accordingly, the transformed cells are grown up to an appropriate stage before expression of the foreign protein is begun and expression is generally considered viable for only a few hours. The situation is quite different for the elastic protein-based polymer considered here. This may result in part due to the extraordinary biocompatibility exhibited by $(GVGVP)_n$ and its related polymers. The elastic protein-based polymer, $(GVGVP)_n$ and its γ-irradiation crosslinked matrix as well as related polymers and matrices appear to be ignored by a range of animal cells and by tissues of the whole animal *(5–7)*. This chapter describes in detail methodologies to accomplish hyperexpression of a protein based polymer in *E. coli*.

2. Materials

2.1. Partial Purification of Polymer Protein

PBS buffer (pH 7.4): 10 m*M* $NaHPO_4$, 2 m*M* KH_2PO_4, 137 m*M* NaCl, 3 m*M* KCl.

2.2. SDS-Gel Electrophoresis and Copper Staining

1. Solution 1: 30% acrylamide (dissolve 58.4 g acrylamide and 1.6 g bis-acrylamide in 200 mL distilled water).
2. Solution 2: 1.5*M* Tris-HCl, pH 8.8.
3. Solution 3: 10% sodium dodecyl sulfate (SDS).
4. Solution 4: 0.5*M* Tris-HCl, pH 6.8.
5. Solution 5: 10% ammonium persulfate (freshly prepared).
6. TEMED (tetramethylethylenediamine).
7. Electrophoresis buffer: Dissolve 12 g Tris, 57.6 g glycine, and 4 g SDS in 4 L of water.
8. 2X Gel loading buffer: 100 m*M* Tris-HCl, pH 6.8, 200 m*M* dithiothreitol (DTT), 4% SDS, 0.2% bromophenol blue, 20% glycerol.

9. High range protein marker (Bio-Rad, Hercules, CA).
10. Staining buffer: 0.19*M* Tris-HCl, pH 8.8 and 0.1% SDS.
11. 0.3*M* $CuCl_2$.

All the stock solutions should be prepared in distilled water. Store solutions 1, 2, 4, and 6 at 4°C and 9 at –20°C. All others solutions may be stored at room temperature.

2.3. Transmission Electron Microscopy

1. 0.2*M* Cacodylate buffer: Dissolve 42.8 g sodium cacodylate in 1 L distilled water, adjust pH to 7.2 by adding concentrated NaOH or HCl.
2. 3% Glutaraldehyde (final concentration) in 0.05*M* cacodylate buffer.
3. 1% Osmium tetroxide (final concentration) in 0.05*M* cacodylate buffer.
4. 2% Agarose: Add 0.2 g agarose in 10 mL water and dissolve by boiling in a microwave.
5. Graded series of ethanol (30, 50, 70, 80, 90, 100%).
6. Propylene oxide.
7. Spurr's low viscosity embedding resin *(8)*.
   a. Vinyl cyclohexane dioxide (VCD [ERL 4206])—20 g.
   b. Diglycidyl ether of polypropylene glycol (DER 736)—12 g.
   c. Nonenyl succinic anhydride (NSA)—52 g.
   d. Dimethyl amino ethanol (DMAE)—0.8 g.
   Add and mix the first three components thoroughly before adding DMAE, then mix again. This mixture can be stored in a refrigerator inside a desiccator.
8. Super glue (Devcon, Wooddale, IL).

Store all solutions at 4°C except propylene oxide.

2.4. Staining of Grids

1. 1% Uranyl acetate, pH 4.0 in double distilled water.
2. Lead citrate, pH 12.0.
3. 0.1*N* NaOH.
4. NaOH electrolytic pellets.

Store solutions 1 and 2 at 4°C.

3. Methods

3.1. Partial Purification of Polymer Protein

1. Pellet cells by centrifugation (5000*g*, 10 min, 4°C) from 48 h TB grown cultures without IPTG induction. Discard supernatant.
2. Wash the pellet twice with 10 mL PBS buffer (pH 7.4).
3. Resuspend pellet in 4 mL PBS buffer.
4. Lyse cells by sonication twice, 15 min each (50% amplitude) or by French Press (1200 psi).
5. Centrifuge to remove cell debris (14,000*g*, 10 min, 4°C).
6. Collect supernatant and distribute into microfuge tubes, 1.5 mL each.

7. Incubate tubes at 37°C for 20 min to allow phase transition of polymer to insoluble form
8. Spin at 5000*g* for 3 min at room temperature to allow settling down of polymer.
9. Discard supernatant and resuspend the pellet in 100 µL PBS buffer.
10. Store tubes on ice for 15 min to allow reverse phase transition to soluble form.
11. Centrifuge at 14,000*g* for 10 min at 4°C.
12. Collect supernatants as partially purified polymer sample.

3.2. SDS-Gel Electrophoresis of the Polymer Protein

Prepare and carry out electrophoresis of SDS-polyacrylamide gels according to Laemmli *(9)*.

1. Lower gel preparation (10%): Add 10 mL solution 1, 7.5 mL solution 2, 0.3 mL, solution 3, 12 mL distilled water, 100 µL solution 5, and 10 µL TEMED into a 100-mL flask and mix well using a transfer pipet. Immediately pour the solution in between gel plates and allow it to polymerize for 30 min.
2. Stacking gel preparation: Add 1.4 mL solution 1, 2.6 mL solution 4, 100 µL solution 3, 5.9 mL distilled water, 50 µL solution 5, and 10 µL TEMED into a 100-mL flask and mix well using a transfer pipet. Immediately pour the solution on the lower gel in between the plates and place the comb in place avoiding air bubbles. Allow it to polymerize for 30 min.
3. Centrifuge 1.5 mL of culture in a microfuge tube at 12,000*g* for 45 s.
4. Remove supernatant and wash the pellet in 500 µL of Tris-HCl (50 m*M*, pH 7.6).
5. Centrifuge again to pellet cells, remove supernatant, and resuspend pellet in 100 µL of water.
6. To a fresh microfuge tube, add 20 µL of above sample and an equal volume of 2X SDS gel loading buffer and boil for 5 min. Also boil the high-range protein marker (2 µL) and partially purified polymer (2 µL) after adding 2X loading buffer.
7. Immediately load 40 µL of each sample into individual wells along with high-range protein marker (Bio-Rad) and partially purified polymer protein.
8. Run the gel at 26 mA for 5 h (the current and the run time can be adjusted according to the gel size and convenience).
9. After electrophoresis, take out the gel and soak in Tris-HCl (0.19*M*, pH 8.8 + 0.1% SDS) for 10 min with gentle shaking.
10. Rinse in distilled water once and soak again in 0.3*M* $CuCl_2$ solution for 5 min on a shaker *(10)*.
11. Observe polymer polypeptides as negatively stained bands against a dark background.

3.3. Transmission Electron Microscopy

1. Wash *E. coli* cells twice with distilled water by centrifuging and resuspending the pellet.
2. Resuspend the pellet after second wash in 5 mL of 3% glutaraldehyde (final concentration) in 0.05*M* cacodylate buffer for 3 h at 4°C.
3. Pellet cells, remove supernatant, and resuspend the pellet in 0.05*M* cacodylate buffer for 12 h to remove excess glutaraldehyde.

4. Pellet cells and resuspend pellet in 5 mL of 1% osmium tetroxide (final concentration) in cacodylate buffered solution (0.05*M*) for 1.5 h.
5. Wash the pellet again with 0.05*M* cacodylate buffer to remove any unbound Os.
6. Pellet cells, remove supernatant and, keep the pellet as dry as possible.
7. Boil 2% agarose solution in a microwave and cool it to 50°C.
8. Add 2 mL of 2% agarose solution to the pellet and mix the pellet and agar with a toothpick, which should form a slurry and solidify in a few minutes.
9. From the solidified pellet, mince about 1 mM^3 size blocks with razor blade and place them in glass vials (~10 pieces per vial).
10. Carry out dehydration steps in glass vials. Add 2 mL of each grade of ethanol (30, 50, 70, 80, 90, and 100%) at an interval of 20 min. Remove the previous grade completely using a transfer pipet before adding the next higher grade of ethanol.
11. Similarly, treat samples with three changes of propylene oxide in 20 min intervals.
12. Add a mixture of propylene oxide:Spurr's resin in the proportion of 3:1. Repeat this step three times by gradually increasing resin content over propylene oxide (1:1, 3:1, and pure resin) in intervals of 2 h each on a gyrator. Finally, add pure resin and allow to infiltrate for 12 h on the gyrator with 2–3 changes of pure resin in between. After 12 h, remove the old resin and add 2 mL of fresh resin.
13. Embed blocks in plastic molds and allow the resin to cure by incubating at 65°C for 8 h.
14. Cut the sample out of the mold using a saw, mount on the resin block using super glue, and incubate at 65°C for 40 min.
15. Trim blocks with glass knives and section using a microtome.
16. Pickup silver sections with a thickness of ~60 nm on copper grids for staining. Steps 1–10 should be done at 4°C.

3.4. Staining of Grids

1. Put a piece of parafilm in a Petri plate. Add drops of uranyl acetate (pH ~4.0) separately on parafilm according to the number of grids to be stained (one drop per grid).
2. Carefully place one grid in each drop and wait for 40 min.
3. Wash each grid in double distilled water by dipping it at least 20X using a pointed forceps and dry on a filter paper.
4. Add a few electrolytic pellets of NaOH in a Petri dish containing parafilm.
5. Add drops of lead citrate (pH ~12.0), place one grid in each drop and wait for 2 min.
6. Wash each grid first in 0.1*N* NaOH, then in double distilled water, and dry the grids on filter paper.

Observe specimens under a transmission electron microscope at 60 kV accelerating voltage.

3.5. Evaluation of Results

Construction of a synthetic protein-based polymer gene: As an illustration of an uninduced hyper-expression of a protein-based polymer in *E. coli*, we have chosen a gene encoding 121 repeats of the elastomeric pentapeptide

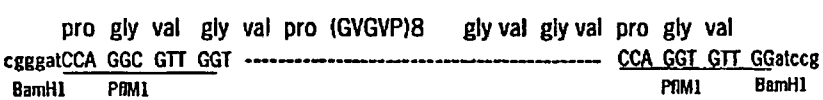

Fig. 1. Amino acid sequence and flanking restriction endonuclease sites of the basic polymer building block coding for $(GVGVP)_{10}$. Using synthetic oligonucleotides and PCR, $(GVGVP)_{10}$ was amplified with flanking BamHI and pflM1 ends and the 121-mer gene was inserted into pUC118 as a BamHI fragment. For expression under control of the T7 polymerase gene promoter, a 121-mer gene was created by concatenation of the PflMI 10-mer fragment with terminal cloning adaptors and subsequently inserted into the expression vector pET-11d.

GVGVP. This gene, $(GVGVP)_{121}$, was constructed by ligase concatenation of a DNA sequence encoding $(GVGVP)_{10}$ and isolation of a concatemer having 12 repeats of this monomer gene plus an additional C-terminal GVGVP sequence encoded by a 3' cloning adaptor *(10a)*. The gene encoding $(GVGVP)_{10}$ was synthesized and cloned into a multipurpose cloning plasmid from which it was then excised by digestion at flanking sites with the restriction endonuclease PflM1 (Fig. 1). A substantial amount of the PflM1 gene fragment was purified and self-ligated in the presence of limited amounts of synthetic double-stranded oligonucleotide adapters that provided the additional restriction sites needed for cloning the resulting concatemers. PflM1 cleaves at its recognition site in the DNA to leave two single-stranded extensions that are not self-complementary (i.e., nonpalindromic) but are only complementary to each other; therefore, proper translational polarity is maintained by head-to-tail tandem coupling of the monomer gene units by ligase during the concatenation reaction.

3.5.1. Vector Construction and Polymer Gene Expression

Concatemer genes recovered by the above procedure, including the $(GVGVP)_{121}$, were ultimately placed into the expression vector pET-11d (Novagen, Madison, WI) immediately adjacent to the initiator ATG codon. This vector is part of the T7 expression system *(11)* that utilizes the coliphage T7 RNA polymerase to drive expression from the T7 promoter on the plasmid. In this case, the polymerase is provided by the host strain HMS 174 (DE3), a lambda lysogen carrying the T7 RNA polymerase gene on the stably integrated phage genome *(12)*. Expression of the RNA polymerase gene is under control of the *lac*UV5 promoter and is therefore inducible by addition of IPTG to the growth medium. Expression of an inserted foreign gene in pET-11d is regulated by two *lacI* repressor genes, one located in the plasmid pET-11d and the other in the genome of the host strain HMS 174 (DE3).

Gene expression was studied in samples each grown in either Luria broth (LB) or in Terrific broth (TB, ref. *13*) in the presence or absence of ampicillin (100 μg/mL) at 37°C. After 3 h of growth (at an OD of 0.8), cultures were induced with 1 mm isopropylthio-β-D-galactoside (IPTG) and continued to grow for different durations; cells were stored at 4°C at the end of the time-course.

Cell lysates of both TB and LB grown cultures separated on SDS-polyacrylamide gels are shown in Fig. 2. Polymer protein can be seen by negative staining around 60 kDa (Fig 2). The pattern of polymer production is observed to be the same in both gels, although the quantity of polymer is several-fold more in TB grown cultures (uninduced). The amount of polymer in uninduced 6 h sample (lane 3) is approximately comparable to that of the induced 6 h sample (lane 4). However, there is a dramatic increase in the expression of polymer in uninduced cultures grown for 24 h (lane 5) over induced cultures of the same age (lane 6). This increase is more pronounced in TB grown cultures compared to LB grown cultures which is not surprising because it is known that in TB grown cultures, copy number of the plasmid increases by four- to sevenfold and the cell density increases by 10-fold over those of the LB grown cultures *(13)*. In contrast, the amount of polymer produced in induced cultures is negligible (lanes 6, 8, and 10) accompanied by irregular shapes of cells (*see* the electron micrograph in Fig. 3C). Decrease in polymer production in induced cells could be directly correlated with loss of the introduced plasmid and reduced cell growth. No plasmid DNA was found in cells induced with IPTG beyond 6 h of growth (data not shown). Reduced cell growth after IPTG induction has been reported earlier. For example, Brosius *(14)* reported that induction of trp/lac (tac) hybrid promoter with 1–5 m*M* IPTG in *E. coli* strain RB 791 (lac repressor overproducing strain) caused reduced cell growth, ultimately leading to cell lysis. Masui *(15)* also reported that the growth rate of *E. coli* T19 cells induced with IPTG was reduced after 5–6 h. In our studies, the highest expression of protein is observed in 24 h uninduced cultures (lane 5), followed by a gradual reduction in cultures grown beyond 24 h (lanes 7 and 9). This may be owing to cell lysis as well as decrease in plasmid copy number after 24 h as evident from light microscopic observations and plasmid DNA isolation studies (data not shown). However, it should be pointed in this context that this polymer protein, $(GVGVP)_{121}$, is extraordinarily stable in *E. coli* cells as seen in Fig. 2 (lane 12) that shows polymer present in a 48 h uninduced culture stored for over three months at 4°C.

The polypeptide observed at the same mol wt as β-galactosidase (116.3 kDa), after 24 h of growth (Fig. 2, lanes 6, 8, 10) in induced cells but not in uninduced cells (Fig. 2, lanes 3, 5, 7, 9) may be β-galactosidase (a chromosomal gene) induced by addition of gratuitous inducer (IPTG). This result is in accordance with earlier reports that bacteria produce β-galactosidase only when

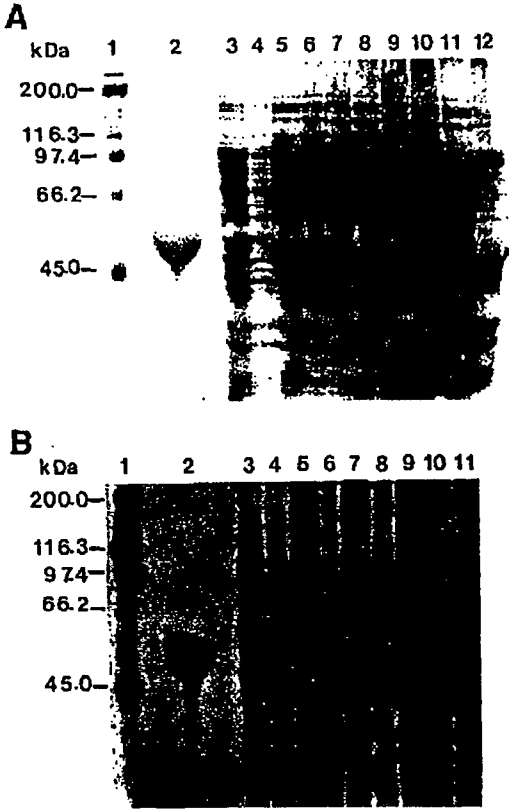

Fig. 2. Crude protein extracts from *E. coli* strain HMS 174 (DE3) transformed with pET 11d-120 mer separated on SDS-PAGE gels. (A) Cultures grown in Terrific Broth (TB). (B) Cultures grown in Luria Broth (LB). Lane 1: high range protein marker showing (top to bottom) myosin, β-galactosidase, phosphorylase b, bovine serum albumin, and ovalbumin; lane 2: partially purified polymer standard; lane 3: uninduced-6 h; lane 4: induced-6 h; lane 5: uninduced-24 h; lane 6: induced-24 h; lane 7: uninduced-48 h; lane 8: induced-48 h; lane 9: uninduced-72 h; lane 10: induced-72 h; lane 11: host strain without plasmid; and lane 12: uninduced-48 h culture stored for 3 mo at 4°C. For induction, 1 m*M* IPTG was added when the cultures reached 0.8 OD.Reprinted from Guda et al. *(3a)*.

its substrate is added to the medium *(16)*. Excess production of polymer in 24 h uninduced cells may also be attributed to reduction or dilution of repressor protein as evidenced by increase in β-galactosidase production in induced cells

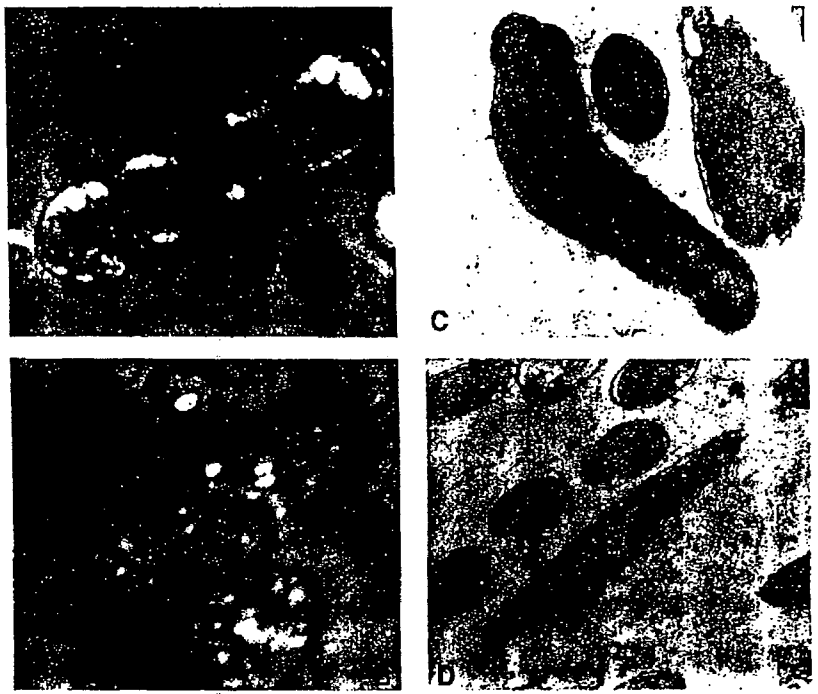

Fig. 3. Transmission electron micrographs of *E. coli* strain HMS 174 (DE3) transformed with pET 11d-121 mer showing polymer production in uninduced and induced cells. (A,B) uninduced cells, 24 h; (C) an induced cell, 24 h; (D) an uninduced cell without plasmid.

of the same age (Fig. 2). Dilution of the repressor protein in rapidly growing cells should have enabled the RNA polymerase to efficiently bind and initiate transcription of the T7 promoter that drives the polymer gene. It is known that the amount of repressor protein produced by DE3 on host chromosome is not sufficient to block the T7 polymerase transcription; BL21 (DE3) cells transformed with the pT7-5 plasmid that carries a *cryIIA* gene driven by the T7 promoter produced large quantities of *cryIIA* crystals without any need for induction with IPTG *(17)*.

Light micoscopic studies using oil immersion lens (Carl Zeiss, plan 100/1, 25 oil ph3) showed distinct intracellular inclusion bodies both in induced and uninduced cells at 6 h growth period (data not shown). The first inclusion body in a cell is found generally but not necessarily at one end of the cell. But, as the

Production and Purification of a Recombinant Elastomeric Polypeptide, G-(VPGVG)$_{19}$-VPGV, from *Escherichia coli*

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An elastomeric polypeptide was produced, with the sequence G-(VPGVG)$_{19}$-VPGV, as a fusion to glutathione *S*-transferase using the vector pGEX-3X. The fusion protein was expressed to high levels in *Escherichia coli* as indicated by SDS–PAGE analysis of induced cells. The fusion protein was affinity purified and cleaved with protease factor Xa, and the elastomeric polypeptide was recovered to a high degree of purity as indicated by SDS–PAGE followed by staining with $CuCl_2$. The physical characterizations of carbon-13 and proton nuclear magnetic resonance and of the temperature profile for turbidity formation for the inverse temperature transition of hydrophobic folding and assembly attest to the successful microbial synthesis of the polypentapeptide of elastin. The results of these studies provide the initial progress toward achieving a more economical and practical means of producing material for elastic protein-based polymer research and applications.

Introduction

Previous characterizations of the biophysical nature of elastomeric polypeptide biomaterials have relied upon chemical synthesis of the materials involved (Urry, 1988, 1991). Chemical syntheses of the repeating peptides of elastin have been quite successful when the repeating unit is sufficiently small, e.g., 3–9 residues, and when the component oligopeptides of the repeat and the repeat itself are carefully purified prior to polymerization using a carboxyl terminus such as Pro or Gly where racemization is not a problem (Urry and Prasad, 1985; Prasad et al., 1985). When the desired polypeptide is more complex as in combining fixed-length blocks of repeats or in preparing sequences within which there are limited repeating sequences, solid-phase synthesis has been attempted with limited success because of the difficult purifications required to remove small amounts of racemization and possibly occasional deletions in these elastic protein-based polymers (unpublished data). Small amounts of such errors can significantly alter the physical properties of elastic moduli and temperatures and heats of the folding transitions, but this occurs in ways that do not lend themselves to useful means of purification.

As an initial demonstration of an alternative preparative method, we have approached the biological production of an elastomeric polypentapeptide, with the basic repeating unit VPGVG (valine-proline-glycine-valine-glycine), using *Escherichia coli* and employing recombinant DNA methodology. This will ultimately provide verification of the temperatures and heats of the hydrophobic transition, of the circular dichroism spectra, of the nuclear magnetic resonance spectra, and of the elastic properties of the chemically synthesized poly(VPGVG) and will demonstrate the methodology for preparing more complex elastic protein-based polymer constructs. In order that the recombinant polypeptide might be effectively produced, detected, and purified without the possibility of a N-terminal formylmethionine residue, it was co-produced as a C-terminal fusion to a protein that allows proteolytic release of the polypentapeptide. The pGEX vectors, described by Smith and Johnson (1988) and available commercially from Pharmacia, provide a suitable means for expressing fusion genes whose products, C-terminal fusions to glutathione *S*-transferase (gst), can be affinity purified by adsorption to glutathione–agarose.

Synthetic oligonucleotides were used to construct a gene which encodes 10 repeating units of the elastomeric pentapeptide VPGVG, i.e., (VPGVG)$_{10}$. Then, using the polymerase chain reaction (PCR) (Saiki et al., 1987) to amplify the sequence, it was subcloned into pGEX-3X to create a gene that expresses G-(VPGVG)$_{19}$-VPGV as a C-terminal fusion to gst. The fusion protein, gst-G-(VPGVG)$_{19}$-VPGV, contains the recognition sequence for protease factor Xa at the fusion junction. The fusion gene was expressed to high levels in *E. coli*, purified, and cleaved from the fusion protein to produce quantities of G-(VPGVG)$_{19}$-VPGV needed for biophysical and chemical studies on the recombinant elastomeric polypeptide.

Materials and Methods

Culture Conditions. *E. coli* strain MV1190 [Δ(*lac-proAB*), *thi*, *supE*, (Δ*srl-recA*)306::Tn10, (F′: *tra*D36, *proAB*, *lacI*^{q}*Z*δM15)] was obtained from Bio-Rad Laboratories and, transformed (Maniatis et al., 1982) with the appropriate plasmid, was used as the host strain in all cultures. All cultures were grown at 37 °C in Luria broth (Maniatis et al., 1982) supplemented with 50–100 μg/mL ampicillin. Fermentation was done in a 16-L New Brunswick fermentor with a final volume of 12 L. Cultures for the production of fusion protein were monitored for growth either with a Klett–Summerson colorimeter with a red no. 66 filter or with spectrophotometric methods by absorbance at 600 nm. Isopropyl β-D-thiogalactopyranoside

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(IPTG), obtained from Sigma Chemical Co., was added to 1 mM for the induction of fusion protein gene expression. Inductions were performed at a cell density corresponding to 90 Klett units for small-scale cultures and at an A_{600} of approximately 3.6 for fermentation-scale cultures. All cultures were harvested 3 h following induction.

Cloning, DNA Preparation, and Sequencing. Standard recombinant DNA techniques were used in the construction of all vectors (Maniatis et al., 1982). Enzymes used for DNA modification, cloning, and analysis were purchased either from Boehringer Mannheim or from Stratagene. Plasmid DNA was prepared by a modified alkaline lysis method (Ish-Horowicz and Burke, 1981), and DNA sequencing was performed by the dideoxy chain-termination method (Sanger et al., 1977) using the Sequenase kit from United States Biochemicals.

Synthetic Oligonucleotides. The universal sequencing primer was obtained from New England Biolabs. All other oligonucleotides either were synthesized on an Applied Biosystems automated DNA synthesizer by the University of Alabama at Birmingham (UAB) Cancer Center DNA Synthesis Core Facility or were purchased from Oligos, Etc.

Construction of Synthetic Gene. A DNA sequence coding for $(VPGVG)_{10}$ was constructed using two synthetic oligonucleotides, each 85 bases in length, with 3′-overlapping complementary ends. They had the following sequences:

5′-GTTCCGGGTGTTGGTGTACCGGGTGTTGGT-
GTGCCGGGTGTTGGTGTTCCGGGCGTAGGC-
GTACCGGGCGTAGGCGTGCCGGGCG-3′

5′-ACCTACACCCGGAACGCCCACACCCGGCACG-
CCCACGCCCGGTACGCCCACGCCCGGAACGC-
CTACGCCCGGCACGCCTACGCCC-3′

Briefly, the 3′ ends were annealed through a 20-base region of complementarity and extended with AMV reverse transcriptase and deoxynucleotides to provide complementary strands of 150 bases.

Polymerase Chain Reaction (PCR). PCR (Saiki et al., 1987) reactions were performed in a total volume of 100 μL containing approximately 1 ng of plasmid DNA as template and 100 pmol of each primer in a mixture of 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 200 mM each deoxynucleotidetriphosphate, and 2.5 units of recombinant *Thermus aquaticus* DNA polymerase (Amplitaq, Perkin-Elmer Cetus). The above mix was overlaid with an equal volume of mineral oil (reagent-grade, Sigma) and subjected to 30 cycles of 94 °C for 1 min, 52 °C for 3 min, and 72 °C for 3 min in a Perkin-Elmer Cetus DNA thermal cycler, with minimal ramp time between steps. In each case, a DNA fragment of the desired size was purified by first digesting the PCR product with the appropriate restriction enzymes, followed by electrophoresis through 6% acrylamide, band excision, electroelution into dialysis tubing, and precipitation with ethanol.

Vector Constructions. pGEX-3X was obtained from Pharmacia, and phage M13mp18 was obtained from New England Biolabs. Plasmid pEPP-1 was constructed by blunt-end cloning the 150-bp synthetic gene coding for $(VPGVG)_{10}$ into the *Sma*I site of M13mp18. The sequence of the synthetic gene was then verified by DNA sequence analysis using the universal primer.

pEPP-2 was constructed by subcloning the PCR-amplified $(VPGVG)_{10}$ sequence into pGEX-3X. The gene was amplified using a 27-mer forward primer, 5′-CTGAATTGGTTCCGGGTGTTGGTGTAC, and a 30-mer reverse primer, 5′-CATGAATTCTTATACACCCGGGACGCCCAC, using pEPP-1 as the template. The subsequent product was digested with *Xmn*I and *Eco*RI and inserted into the S1 nuclease treated *Bam*HI and the *Eco*RI sites of pGEX-3X.

	V	P	G	V	G	V	P	G	V	G	
5′ –	GTT	CCG	GGT	GTT	GGT	GTA	CCG	GGT	GTT	GGT	
3′ –	CAA	GGC	CCA	CAA	CCA	CAT	GGC	CCA	CAA	CCA	
	GTG	CCG	GGT	GTT	GGT	GTT	CCG	GGC	GTA	GGC	
	CAC	GGC	CCA	CAA	CCA	CAA	GGC	CCG	CAT	CCG	
	GTA	CCG	GGC	GTA	GGC	GTG	CCG	GGC	GTA	GGC	
	CAT	GGC	CCG	CAT	CCG	CAC	GGC	CCG	CAT	CCG	
	GTT	CCG	GGC	GTG	GGC	GTA	CCG	GGC	GTG	GGC	
	CAA	GGC	CCG	CAC	CCG	CAT	GGC	CCG	CAC	CCG	
	GTG	CCG	GGT	GTG	GGC	GTT	CCG	GGT	GTA	GGT	– 3′
	CAC	GGC	CCA	CAC	CCG	CAA	GGC	CCA	CAT	CCA	– 5′

Figure 1. Sequence of the synthetic gene coding for $(VPGVG)_{10}$. The gene, with blunt termini, was inserted into M13mp18 at the *Sma*I site to create pEPP-1.

pEPP-3 was constructed by cloning another copy of the $(VPGVG)_{10}$ sequence into pEPP-2. PCR was used to amplify the gene sequence from pEPP-2. A 28-mer forward primer, 5′-AGGTGTAGGTGTTCCGGGTGTTGGTGTA, was used in addition to the 30-mer reverse primer described above. The product of amplification was treated with Klenow to remove any 3′-protruding nucleotides, digested with *Eco*RI, and inserted into the *Sma*I and *Eco*RI sites of pEPP-2. Cloned sequences in both pEPP-2 and -3 were verified by DNA sequencing using both forward and reverse primers synthesized with complementarity to sequences flanking the cloning sites in pGEX-3X.

Protein Gel Electrophoresis and Quantitation. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) of proteins was done using the method of Laemmli (1970) with either a 12.5, 15, or 20% acrylamide resolving gel. Following electrophoresis, proteins were visualized by staining with Coomassie Blue or by negative staining with 0.3 M $CuCl_2$ (Lee et al., 1987). Molecular weight markers were from Bio-Rad Laboratories. Protein concentrations, except as noted below, were estimated by the dye-binding assay of Bradford (1976) using reagents supplied in kit form from Bio-Rad, with bovine serum albumin as the standard. Concentration of the final protein product, G-$(VPGVG)_{19}$-VPGV, was determined by absorbance at 205 nm using the extinction coefficient (E = 2370) calculated from the absorbance of a known quantity of chemically synthesized poly(VPGVG).

Purification of the gst–G-$(VPGVG)_{19}$-VPGV Fusion Protein. Methods for affinity purification of glutathione *S*-transferase-based fusion proteins produced using pGEX vectors have been described (Smith and Johnson, 1988). Glutathione-linked Sepharose was obtained from Pharmacia. Fusion protein was purified from the fermentation culture after concentration of the 12 L to about 1 L using a Millipore tangential flow filtration unit, followed by centrifugation into four equal cell pellets. Each cell pellet was resuspended with 50 mL of PBST (150 mM NaCl, 16 mM Na_2HPO_4, 4 mM NaH_2PO_4, 1% Triton X-100), and cells were lysed by sonic disruption using a Heat Systems XL sonicator. Following removal of the cell debris by centrifugation (25 min at 10000*g*), the sonicate supernatant was passed slowly through glutathione–Sepharose in a 1 × 10 cm column (econo-column, Bio-Rad Laboratories) with a 6-mL bed volume. The column was washed with 3 volumes of PBST, and the bound fusion protein was eluted and collected in 2-mL fractions with approximately 2 volumes of 10 mM glutathione in PBST. The column was then washed with PBST, and the sonicate supernatant

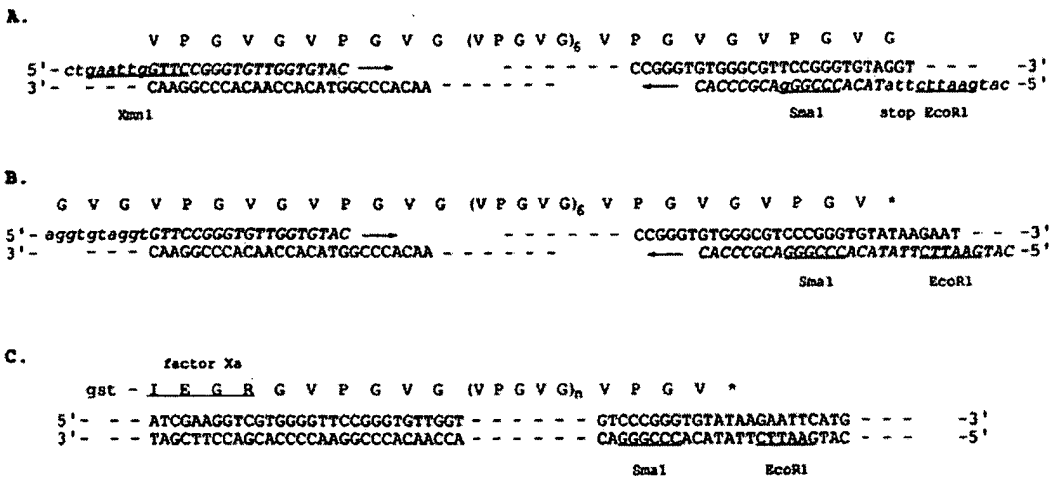

Figure 2. PCR primers, in italics, shown with the complementary sequences to which they were annealed. Changes to the template sequence and other noncomplementary primer sequences are in lowercase letters. (A) The synthetic gene in pEPP-1 was used as the template for amplification of the elastomeric coding sequence for cloning as a *Xmn*I–*Eco*RI fragment into the S1 nuclease treated *Bam*HI and the *Eco*RI sites of pGEX-3X. The resulting plasmid, pEPP-2, directs the expression of G-(VPGVG)$_9$-VPGV as a C-terminal fusion to gst. (B) pEPP-2 was used as the template for amplification of the sequence for cloning as a blunt–*Eco*RI fragment. This fragment was then inserted into the *Sma*I–*Eco*RI sites of pEPP-2, resulting in pEPP-3 and expanding the elastomeric sequence to G-(VPGVG)$_{19}$-VPGV. (C) A representation of the gst–polypentapeptide fusion in pEPP-2 (n = 8) and pEPP-3 (n = 18) showing the factor Xa recognition site at the C-terminus of gst.

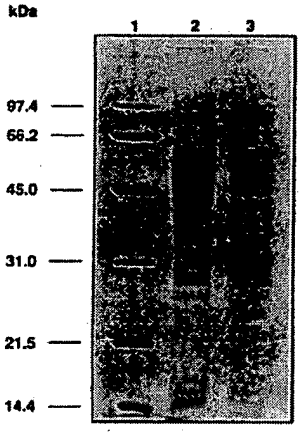

Figure 3. SDS–PAGE of whole-cell lysates of fermentation culture taken just prior to, and 3 h following, induction with 1 mM IPTG. The gel, stained with Coomassie Blue, indicates the production of a protein, following induction, that is the appropriate size (approximately 36 kDa) for the gst–G-(VPGVG)$_{19}$-VPGV fusion. Samples were loaded at 3 Klett unit equivalents (Klett-mL) per lane.

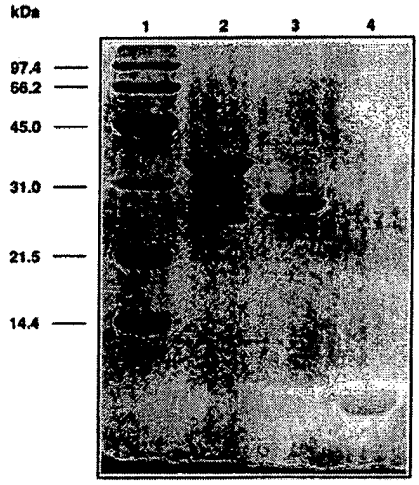

Figure 4. Copper-stained gel showing the following: (1) molecular weight markers; approximately 4 μg of gst–G-(VPGVG)$_{19}$-VPGV fusion protein (2) before and (3) after cleavage with factor Xa; (4) 10 μg of purified G-(VPGVG)$_{19}$-VPGV. Note the opaque "halo" surrounding the elastomeric polypeptide band.

was reapplied for a second binding and elution. Fractions containing the eluted fusion protein were pooled and concentrated either by (1) dialysis against poly(ethylene glycol) (MW 20 000) in NTC [100 mM NaCl, 50 mM Tris-HCl (pH 8.0), 1 mM $CaCl_2$] followed by dialysis against NTC alone or by (2) precipitation with ammonium sulfate at 75% of saturation followed by resuspension in, and dialysis against, NTC. To remove the Triton X-100 detergent, the concentrated fusion protein resulting from each of the four cell pellets was pooled and stirred for 6 h with Calbiosorb (amount according to stated binding capacity), a hydrophobic resin obtained from Calbiochem.

Protease Xa Digestion and Purification of the Elastomeric Polypeptide. Cleavage of the fusion protein to release the G-(VPGVG)$_{19}$-VPGV elastomeric polypeptide from the gst moiety was accomplished by digestion with protease factor Xa purchased from Boehringer Mannheim. The protease was added to the concentrated fusion protein at a ratio (w/w) of approximately 1:500 and allowed to digest for 5 days with mild stirring at 4 °C. Cleavage of the fusion protein was monitored by SDS–PAGE analysis. The cleavage product was then passed through a glutathione–Sepharose column to remove the gst moiety. The G-(VPGVG)$_{19}$-VPGV was further purified by precipitation with ammonium sulfate at 30% of saturation, followed by resuspension in, and dialysis against, deionized H_2O using an inner bag–outer bag technique. Briefly, the resuspended product was placed in an inner bag of 12 000–14 000-MW cutoff (6.4-mm diameter) and then into an outer bag of 3500-MW cutoff (28.6-mm diameter) with a liquid volume ratio of 1:10. Following dialysis, the G-(VPGVG)$_{19}$-VPGV was recovered from both outer and inner bags, due to the fact that the majority of the upper molecular weight contaminants precipitated in the inner bag and could be removed by centrifugation. The purified polypeptides was then lyophilized and weighed to determine the final yield.

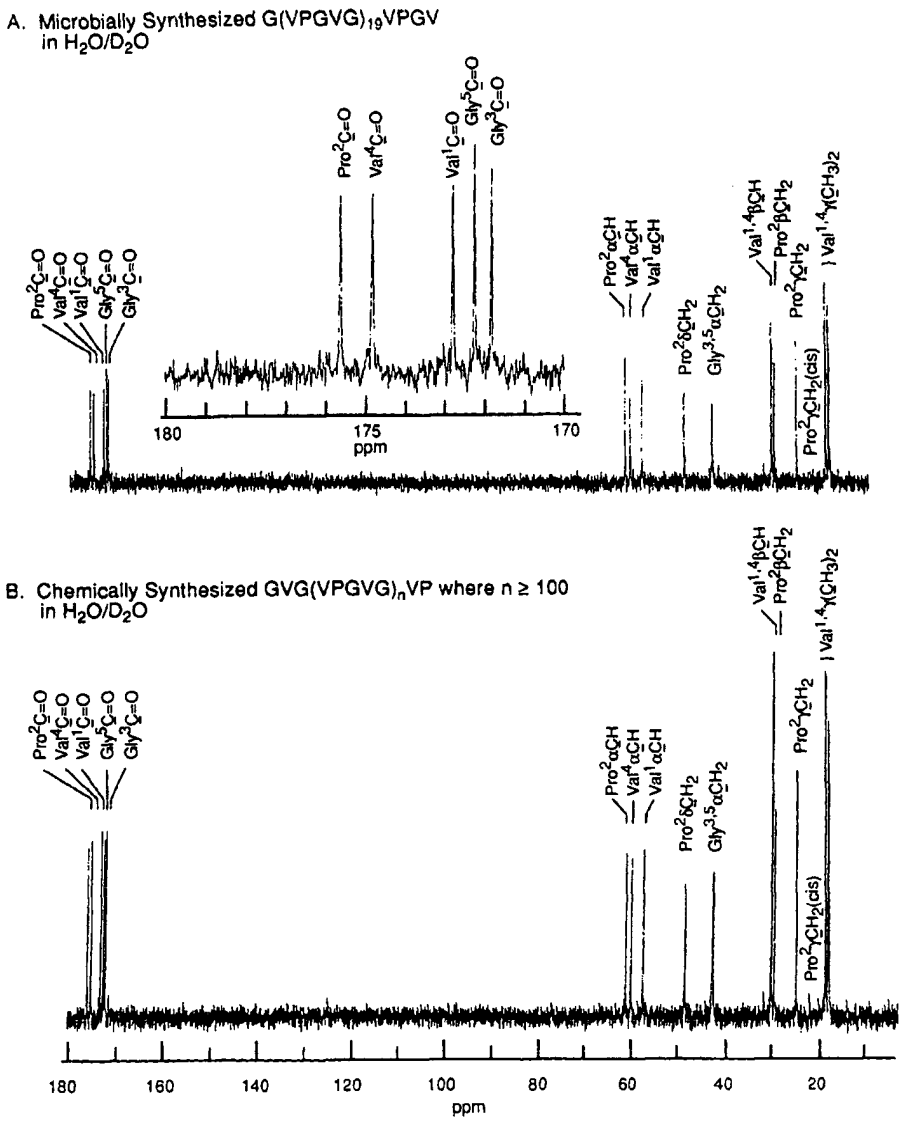

Figure 5. Carbon-13 nuclear magnetic resonance spectra at 100 MHz in H_2O/D_2O. (A) Microbially synthesized G-$(VPGVG)_{19}$-VPGV. The carboxyl region is expanded so as to better look for minor resonances. (B) Chemically synthesized poly(VPGVG) or GVG-$(VPGVG)_n$-VP where n is greater than 100. The successful microbial synthesis and purification are apparent.

Physical Characterization of Recombinant G-$(VPGVG)_{19}$-VPGV. The carbon-13 and proton nuclear magnetic resonance data were obtained on a Bruker WH-400 (9.4 T) spectrometer equipped with an Aspect 3000 computer. The carbon-13 NMR data were obtained at 100 MHz using 6128 pulses and 6.0-μs pulse width. The 400-MHz proton NMR data were obtained using a 4.0-μs pulse width and 256 pulses for the microbially produced sample (20.6 mg/mL) and a 6.0-μs width and 64 pulses for the chemically synthesized sample (23.5 mg/mL). The temperature profile for turbidity formation gives the onset for the folding and aggregational transition by following the turbidity development at 300 nm using the Aviv modification of the Cary Model 14 spectrophotometer. The sample chamber was fitted with a 300-Hz vibrator to minimize settling during the scan.

Results and Discussion

Construction of the gst-G-$(VPGVG)_{19}$-VPGV Expression Vector. To create the gst-G-$(VPGVG)_{19}$-VPGV fusion gene, a gene coding for $(VPGVG)_{10}$ was first constructed by annealing two 85-mer synthetic oligonucleotides through a 20-base region of complementarity at their 3′ termini, followed by chain extension with AMV reverse transcriptase to create a 150-bp double strand. The complete nucleotide sequence of the $(VPGVG)_{10}$ gene is shown in Figure 1. The sequence was designed to allow maximal coding redundancy while *E. coli* codon preference was maintained (Gouy and Gautier, 1982). The 150-bp fragment was inserted into the *Sma*I site of M13mp18 to create the vector pEPP-1. The $(VPGVG)_{10}$ gene was then amplified from pEPP-1 by PCR using forward and reverse primers, as illustrated in Figure 2A. The PCR product was digested with *Xmn*I and *Eco*RI and inserted between the S1 nuclease treated *Bam*HI (blunt) site and the *Eco*RI site of pGEX-3X. The resulting plasmid, pEPP-2, directs the expression of a gst-G-$(VPGVG)_9$-VPGV fusion gene whose product can be affinity purified with glutathione-Sepharose and cleaved with factor Xa. pEPP-2 was used for a second round of PCR amplification as shown in Figure 2B. The G-$(VPGVG)_9$-VPGV coding sequence was amplified, digested with *Eco*RI, and cloned into the *Sma*I-

*Eco*RI sites of pEPP-2 to give the final vector, pEPP-3, containing the fusion gene for gst–G-(VPGVG)$_{19}$-VPGV.

Production of Recombinant G-(VPGVG)$_{19}$-VPGV. The expression of recombinant gst-fusion proteins from pGEX vectors is controlled by the *tac* promoter (DeBoer et al., 1983; Amann et al., 1983) and is induced by the addition of IPTG to the growing culture. Cultures of MV1190 cells, transformed with pEPP-3, were induced at a mid-log stage of growth, and the production of gst–G-(VPGVG)$_{19}$-VPGV fusion protein was analyzed by SDS–PAGE. Coomassie-stained gels of whole-cell lysates prepared just prior to, and 3 h following, induction (see Figure 3) indicate the production of a protein with an apparent molecular weight of ~36 000 following the addition of IPTG; this is the expected size according to the molecular weight of gst, 28 000, and the calculated molecular weight of (VPGVG)$_{20}$, 8.2 000.

In preliminary studies, the digests of purified gst–G-(VPGVG)$_{19}$-VPGV with factor Xa had indicated that cleavage occurred with near equal efficiency whether the fusion protein was still attached or was eluted from the glutathione–Sepharose (data not shown). However, we decided to add Xa to the gst–G-(VPGVG)$_{19}$-VPGV following elution from the Sepharose beads, as this would allow digestion of the fusion protein at concentrations, and amounts, not limited by the binding capacity of the affinity substrate. The cleavage reaction, following the addition of protease Xa to gst–G-(VPGVG)$_{19}$-VPGV, was monitored by visualizing aliquots with SDS–PAGE and Coomassie Blue. These gels showed the gradual decrease of the ~36-kDa fusion protein and increase of the 28-kDa gst; however, the appearance of the 8.2-kDa G-(VPGVG)$_{19}$-VPGVG polypeptide could not be visualized by staining due to its lack of aromatic side chains. To visualize the G-(VPGVG)$_{19}$-VPGV by SDS–PAGE, the negative staining technique described by Lee et al. (1987) was used, whereby the gel was stained with copper (0.3 M $CuCl_2$) resulting in a "negative image" of the electrophoresed proteins. Figure 4 shows an example of a copper-stained gel with samples of the purified gst–G-(VPGVG)$_{19}$-VPGV fusion protein before and after cleavage by factor Xa and a sample of the final product, G-(VPGVG)$_{19}$-VPGV.

Production of material for use in studies involving the physical and chemical characteristics of the recombinant elastomeric polypeptide, G-(VPGVG)$_{19}$-VPGV, was achieved using affinity-purified fusion protein from a 12-L fermentation culture. An estimated quantity of 154 mg of fusion protein was purified from the fermentation cell pellet; of this, an estimated 128 mg was digested with protease factor Xa. The elastomeric moiety was purified from approximately 120 mg of the digest reaction resulting in a final yield of 13.8 mg of G-(VPGVG)$_{19}$-VPGV. Aliquots not carried through the entire purification scheme were removed for further developmental studies.

The carbon-13 NMR spectrum is shown in Figure 5, where all of the signals are assigned (Urry and Long, 1976) and where comparison is made with the chemically synthesized poly(VPGVG). The polypentapeptide has clearly been produced in *E. coli*, and there is little evidence of impurity once chain end-effects are accounted for. Also in the carbon-13 NMR spectrum, there is no significant evidence for the presence of a *cis*-Val–Pro bond which would be most easily seen between 20 and 25 ppm for the *cis*-γ-CH_2 resonance. The insert shows an expansion of the carbonyl region where end residues would be signals seen at a 5% intensity relation to the Pro resonance.

In order to look more carefully for end effects, for evidence of the *cis*-Val–Pro bond, and for impurities, the

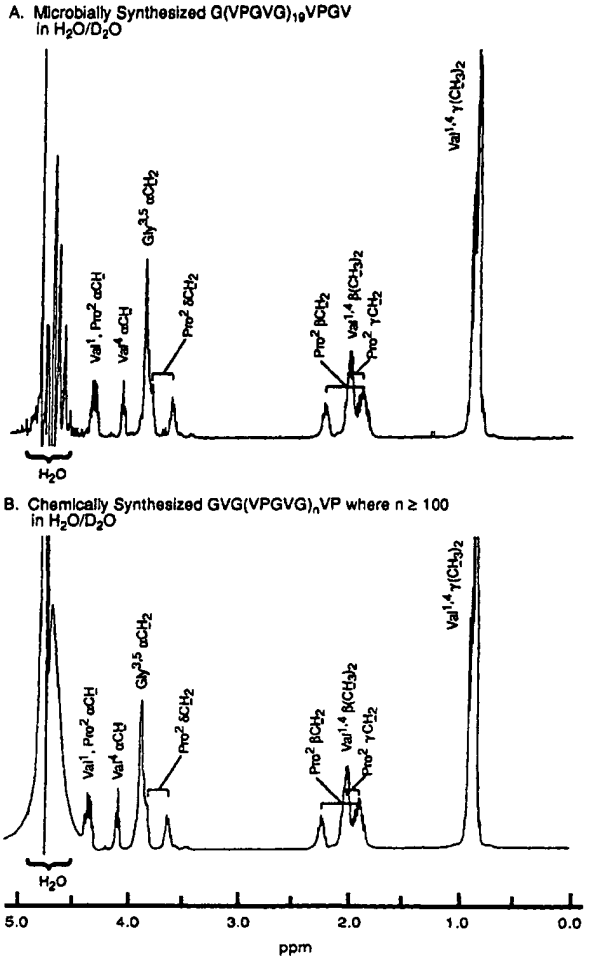

Figure 6. Proton nuclear magnetic resonance spectra at 400 MHz in H_2O/D_2O. (A) Microbially synthesized G-(VPGVG)$_{19}$-VPGV. The minor broad trace resonances are also present in the chemically synthesized polypentapeptide. (B) Chemically synthesized poly(VPGVG) or GVG-(VPGVG)$_n$-VP where n is greater than 100. Any trace resonances present in (A) but not in (B) appear to be due to end effects or low molecular weight solvent impurities. See text for further discussion. Again, the successful microbial synthesis and purification are demonstrated.

proton magnetic resonance spectra are given in Figure 6 at high vertical gain for both the microbial products and the chemically synthesized polypentapeptide. Under these circumstances, there can be seen very minor trace resonances which are common to both the chemical and microbial products and are considered to be due to a similar amount of *cis*-Val–Pro. The remaining trace resonances would be due to the end effects and due to some impurities, e.g., chemicals used in the purification. The trace resonances present only in the microbial synthesis are a multiplet between the Val$^4\alpha CH$ and Gly$^{3,5}\alpha CH_2$ peaks (likely a terminal residue), a doublet near 3.7 ppm, a second doublet near 1.3 ppm, and what appears to be a singlet near 2.7 ppm. The latter three trace resonances due to their sharpness most likely arise from low molecular weight solvent impurities. The peaks in the 4.5–5.0 ppm range are due to the ringing from the water protons. There is no significant evidence for the gst fusion protein or any other protein remaining as a contaminant.

Another characterization, seen in Figure 7, is the temperature profile for turbidity formation which gives the temperature for the onset of folding and aggregation with increase in temperature (Urry et al., 1985). At 40 mg/mL

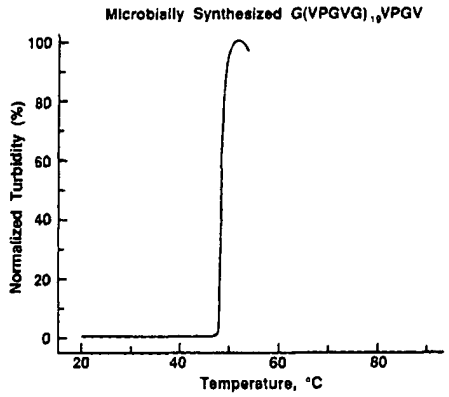

Figure 7. Temperature profile for aggregation (turbidity formation) of microbially synthesized G-$(VPGVG)_{19}$-VPGV. The clean sharp profile indicates a pure sample of uniform molecular weight. See text for discussion.

the temperature is 48 °C for this $n = 20$ polypentapeptide with free α-amino (NH_3^+) and free α-carboxyl (CCO^-) groups (Urry, 1991). The effects of these charges and of the value for n of 20 cause the transition temperature to be higher than for $(VPGVG)_n$, where n is of the order of 120. The profile demonstrates a sharp and simple transition as expected for a pure sample and as would be enhanced by a uniform chain length (Urry, 1988, 1991).

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